U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

MSc

MSc

2.º
CICLO

FCUP
ICBAS
IBMC
2014

U.PORTO

Biotechnological and clinical potential of AIP56 toxin

Inês Salvador Rodrigues

FC

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

INSTITUTO DE CIÊNCIAS
BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

IBMC
INSTITUTO DE BIOLOGIA MOLECULAR E CELULAR
INSTITUTE FOR MOLECULAR AND CELL BIOLOGY

Biotechnological and clinical potential of AIP56 toxin

Inês Salvador Rodrigues

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto e ao
Instituto de Ciências Biomédicas Abel Salazar em
Bioquímica

2014

MSc

MSc

Biotechnological and clinical potential of AIP56 toxin

Inês Salvador Rodrigues
Mestrado em bioquímica
Departamento de Química e Bioquímica
2014
Orientador
Doutor Nuno dos Santos, Investigador auxiliar, IBMC
Coorientador
Doutora Ana do Vale, Investigadora auxiliar, IBMC
Rute Pinto, Investigadora de pós-doutoramento, IBMC

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

INSTITUTO DE CIÊNCIAS
BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

IBMC
INSTITUTO DE BIOLOGIA MOLECULAR E CELULAR
INSTITUTE FOR MOLECULAR AND CELL BIOLOGY

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

AKNOWLEDGMENTS

Um agradecimento a todos o que tornaram possível a execução deste trabalho! Primeiramente ao Doutor Nuno Santos pela oportunidade de estagiar no grupo. Pela orientação e correções que melhoraram o meu trabalho. À doutora Ana do Vale pelo acompanhamento e disponibilidade em discutir o meu trabalho.

Um agradecimento especial à Rute pela paciência, dedicação e a forma incansável como me acompanhou no trabalho laboratorial. Sem dúvida, um exemplo de profissionalismo! Pela serenidade que transmite e pelos conselhos. Um gigante obrigada!

Ao Martinho, que me acompanhou nesta jornada. Pela incansável ajuda ao longo deste ano. Só me resta dizer obrigada!

À Marisa pelo acompanhamento do meu trabalho e disponibilidade em discutir os resultados, pelos conselhos e toda a ajuda ao longo deste ano. Pela boa disposição e gargalhadas que tornaram os dias de trabalhos mais animados.

À minha família por todo o carinho e amizade. Aos meus pais, por serem uma referência e por todas as oportunidades. Pelos ensinamentos, força, carinho e o apoio incondicional que têm demonstrado. À minha irmã que apesar de longe, está perto e que sempre me acompanhou no meu percurso com a maior amizade e carinho. Por sempre acreditar em mim e pela força que me transmite. Ao meu irmão pela amizade que nutrimos.

Aos meus amigos, que sempre acreditaram em mim e me acompanharam com um grande carinho. Joana Ferreira, Rita Costa, Pedro Paiva… vocês sabem o quanto são importantes. Às vezes as palavras tornam-se escassas. Obrigada por estes últimos anos!
Aos que me acompanharam neste percurso académico, pela partilha e carinho que nutrimos: Filipa Sousa, Isabel, Renata, Pedro Araújo, Cleide, Cris e Catarina. À Leninha pela ajuda, carinho e amizade. Pelas gargalhadas contagiantes! À Sara pela amizade.
Aos amigos do Padrão que mesmo ausentes estão sempre presentes.
A todos um muito obrigada! Sem vocês, isto não seria possível.

Este trabalho foi financiado por fundos FEDER através do Programa Operacional de Competitividade - COMPETE e por Fundos Nacionais através da FCT - Fundação para a Ciência e a Tecnologia no âmbito do projeto FCOMP-01-0124-FEDER-028364 (PTDC/BIA-MIC/3463/2012)

ABSTRACT

AIP56 (Apoptosis Inducing Protein of 56 kDa) is a toxin secreted by *Photobacterium damselae* ssp *piscicida* (*Phdp*), a gram-negative pathogen that infects and causes high mortality in marine fish species. The toxin causes selective death of macrophages and neutrophils by post-apoptotic secondary necrosis, being a key virulence factor of *Phdp.* AIP56 is an AB-type toxin, with a N-terminal A domain, which displays metalloprotease activity by cleaving NF-κB p65, connected by a disulphide bridge to a C-terminal B domain, involved in the binding and internalization of the toxin into the host cells. This structural arrangement gives AIP56 a potential to be used as a biomedical, pharmaceutical and biotechnological tool as already happens with other AB-type toxins, such as diphtheria and botulin. Given the important role and involvement of NF-κB in proliferation, cell death and immune responses and the fact that its uncontrolled behavior is associated with human pathologies, such as cancer and degenerative and inflammatory diseases, greatly potentiates the clinical interest of using AIP56 as a biomedical tool.

The present project was aimed at producing AIP56 chimeric proteins that could be directed to cells other than macrophages through replacing the AIP56 receptor-binding domain by protein ligands, whose receptors are over-expressed in cancer cells, and thus, promoting specific targeting, internalization, and intoxication towards determined cancer cells. As a specific purpose, two chimeric proteins were engineered by replacing the putative receptor binding domain of AIP56 (G374 to N497) by either α-Melanocyte Stimulating Hormone (α-MSH) or InterLeukin-3 (IL-3), receptors that are overexpressed in melanoma and acute myeloid leukemia (AML) cells, respectively.

Using molecular biology methods, we were able to clone the DNA sequences encoding each chimeric protein into a proper expression bacterial strain. Production of AIP56-IL3 recombinant protein was then attempted by screening and optimizing known protocols. Despite this, the recombinant protein was always obtained in insoluble state and, therefore, efforts were directed to develop refolding protocols. A successful approach has been developed and a soluble refolded chimera produced. The correct folding of the AIP56 domain was confirmed by *in vitro* testing of NF-κB p65 cleavage, however, it remains to be elucidated whether the IL3 domain has been properly folded.

Functional assays using human AML cell lines for testing whether AIP56-IL3 is able to induce NF-κB p65 cleavage and cell death were performed. As neither p65 cleavage nor apoptosis have been observed, it remains to be clarified whether this has been due to improper folding of the IL3 domain, precluding binding and internalization of the chimera into the cells, deficiency on the translocation process or to a not yet identified reason. Nevertheless, NF-κB p65 cleavage was observed when the AIP56 catalytic

domain was introduced into the cell cytosol of AML cells using the anthrax's LF/PA system as a delivery tool, although it did not result in apoptosis of AML cells.

Key words: AB toxin, AIP56, NF-κB, cell death, leukemia, biotechnological tool

RESUMO

AIP56 (proteína indutora de apoptose de 56 kDa) é uma toxina secretada por *Photobacterium damselae* ssp *piscicida* (*Phdp*), uma bactéria patogénica gram-negativa que infecta e causa elevada mortalidade em espécies de peixes marinhos. A toxina provoca a morte seletiva de macrófagos e neutrófilos por necrose secundária pós-apoptótica, sendo um fator de virulência chave de *Phdp.* A AIP56 é uma toxina do tipo AB, com um domínio A N-terminal que possui atividade de metaloprotease e cuja atividade catalítica é exercida por clivagem da subunidade p65 do NF-κB, ligado por uma ponte dissulfureto ao domínio B C-terminal, que está envolvido na ligação e internalização da toxina nas células do hospedeiro. Este arranjo estrutural confere à AIP56 potencial para ser usada como ferramenta biomédica, farmacêutica e/ou biotecnológica, como já acontece com outras toxinas do tipo AB, tais como as toxinas da difteria e do botulismo. Dado o papel importante do NF-κB na proliferação, morte celular, e resposta imune, assim como o facto de o seu descontrolo estar associado a patologias humanas, como o cancro e doenças inflamatórias e degenerativas, potencia significativamente o interesse clinico de utilização da AIP56 como uma ferramenta biomédica.

O presente projeto teve como objetivo produzir proteínas quiméricas AIP56 de modo a direcioná-las para células, que não macrófagos, através da substituição do domínio de ligação ao recetor da AIP56 por ligandos proteicos, cujos recetores estão sobre-expressos em células cancerígenas, promovendo assim o direcionamento especifico, internalização e intoxicação em determinadas células cancerígenas. Para este fim, duas proteínas quiméricas foram construídas pela substituição do domínio putativo de ligação à célula da AIP56 pela α-hormona estimuladora de melanócitos (α-MSH) ou pela interleucina-3 (IL-3), cujos recetores estão sobre-expressos em células de melanoma e leucemia mieloide aguda (LMA), respetivamente.

Usando métodos de biologia molecular, clonamos as sequências de ADN codificantes para cada uma das proteínas quiméricas, para posterior expressão numa estirpe bacteriana. A produção da proteína recombinante AIP56-IL3 foi então tentada por *screening* e otimização de protocolos conhecidos. Uma vez que a proteína foi sempre obtida na forma insolúvel, foram utilizados protocolos de *refolding* na tentativa de a solubilizar. Uma das abordagens resultou na obtenção de proteína quimérica solúvel. A clivagem *in vitro* da subunidade p65 do NF-κB confirmou que o domínio catalítico da AIP56, no contexto da quimera, tem a conformação correta. No entanto, o mesmo não foi confirmado para o domínio IL3.

Ensaios funcionais usando linhas celulares humanas de LMA para testar a capacidade da AIP56-IL3 clivar a subunidade p65 do NF-κB e induzir apoptose foram realizados. Não foi observada clivagem do p65 nem apoptose, permanecendo por clarificar se este facto se deveu à incorreta conformação do domínio IL-3, impedindo deste modo a ligação e internalização da quimera nas células, se foi devido a deficiência na translocação da toxina do compartimento endossomal para o citosol ou se devido a razão não identificada. Contudo, e alternativamente, foi observada clivagem da subunidade p65 do NF-κB quando se fez chegar ao citosol da células LMA o domínio catalítico da AIP56 usando o sistema antraz FL/AP (fator letal/antígeno protetor), embora não tenha resultado em apopotose das células.

Palavras chave: Toxina AB, AIP56, NF-κB, morte celular, leucemia, ferramenta biotecnológica

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABREVIATION LIST

ADP: Adenosine diphosphate
Arf6: ADP-ribosylation factor 6
AML: Acute Myeloid Leukemia
BAFF: B-cell activating factor
BCL-3: B-cell lymphoma 3
BCR: B-cell receptors
BoNT: Botulinum neurotoxins
cAMP: Cyclic adenosine monophosphate
CC: Coiled-coil
CDT: Cytolethal distending toxin
cGMP: Cyclic guanosine monophosphate
CMG2: Capillary morphogenesis protein 2
CT: Cholera toxin
CTCL: Cutaneous T cell lymphoma
DD: Death domain
DiD: Dimerization domain
dsFv: disulfide-stabilized fragment variable
dNTP: Deoxynucleotide triphosphates
DT: Diphtheria toxin
DTT: Dithiothreitol
EDTA: Ethylenediaminetetraacetic acid
EF: Edema factor
EF-2: Elongation factor 2
EGFR: Epidermal growth factor receptor
ER: Endoplasmic reticulum
FBS: Fetal bovine serum
FDA: Food and Drug Administration
FR β: Folate receptor β
Gb3: Glycosphingolipid
GC-C: Guanylate cyclase C
GM1: Ganglioside
GvHD: Graft versus Host Disease
GRR: Glycine-rich region
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLH: Helix loop helix

IKK: IκB kinase

IL: Interleukin

IL R: Interleukin receptor

IT: Immunotoxins

IPTG: Isopropyl β-D-1-thiogalactopyranoside

Kan: Kanamycin

LB: Lysogenic Broth

LF: Lethal factor

LPS: Lipopolysaccharide

LTβ: Lymphotoxin β

LTβR: Lymphotoxin β receptor

LZ: Leucine-zipper

Mab: Monoclonal antibody

MAPKK: Mitogen activated protein kinase kinase

mBMDM: mouse bone marrow derived macrophages

MTR1: Melanotropin receptors

MHC-II: Major Histocompatibility Complex class II

MSH: Melanocyte-stimulating hormone

NBD: NEMO binding domain

NF-κB: Nuclear factor-κB

NIK: NF-κB-inducing kinase

NLS: Nuclear localization signal

NSCLC: non-small cell lung cancer

NTD: N-terminal domain

PA: Protective antigen

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PE: *Pseudomonas* exotoxin A

PFTs: Pore-forming toxins

Phdp: Photobacterium damselae piscicida

PMSF: Phenylmethanesulfonyl fluoride

PPIase: Peptidylprolyl isomerase

PRRs: Pattern-recognition receptors

RA: Rheumatoid arthritis

RANKL: Receptor activator for NF-κB ligand

RHD: Rel homology domain

RIPs: Receptor interacting proteins

sb: Sea bass

SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis

scFv: Single chain variable fragment

SNAP-25: Synaptosomal-associated protein 25

ST: Shiga toxin

STs: Heat-stable toxins

TAD: Transactivation domain

TBS: Tris-Buffered saline

TCR: T-cell receptors

TeNT: Tetanus neurotoxin

TfR: Transferrin receptor

TLRs: Toll-like receptors

TEM8: Tumor endothelium marker 8

TNF: Tumour necrosis factor

TNFRs: TNF receptors

TRAF: TNF-receptor associated factor

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

UV: Ultraviolet

VAMP: Vesicle-associated membrane protein

VEGF: Vascular endothelial growth factor

ZF: Zinc finger

CHAPTER I

INTRODUCTION

1. Bacterial toxins

In order to successfully colonize their host, many bacterial pathogens owe their virulence to the production of specific proteins called toxins [25]**.**

Since diphtheria toxin was isolated by Roux and Yersin in 1888 [26], microbial toxins have been recognized as the primary virulence factor(s) for a variety of pathogenic bacteria [27]. In microbiology, they have been defined as soluble substances, result of the microbial metabolism, that are lethal to host cells or affect their function negatively even at very low concentrations [27]. Generally, their production is specific to a particular bacterial species (e.g. only *Clostridium tetani* produces tetanus toxin; only *Corynebacterium diphtheria* produces the diphtheria toxin) [28] and they are the major determinant of the virulence of the strains because, usually, virulent strains of the bacterium produce the toxin while nonvirulent strains do not [28].

As virulence factors, they play a major role in the pathogenesis of infectious diseases in the host. These proteins are some of the most powerful human poisons known and retain high activity in the outcome of the infection, contributing to the main lesions and clinical symptoms of the corresponding diseases [27]. Therefore, understanding the mechanism of cell intoxication leads both to the knowledge of the molecular pathogenesis and to the discovery of new aspects of cell physiology [29]. This basic knowledge has resulted in many applications: i) toxins are powerful tools for gaining insights into fundamental processes of cell biology; ii) several toxins are now used to combat several diseases such as cancer, inflammatory or auto-immune diseases [29, 30].

The different types of toxins produced by a bacterial cell can be broadly classified as endotoxins or exotoxins [30]. Endotoxins are part of Gram-negative bacteria, associate with outer membrane or the cell wall, as the lipopolysaccharide (LPS), and are generally available for action only after the death and lysis of the bacteria to which they belong [28]. Most of the protein toxins are thought as exotoxins, which are mostly proteins that are secreted by both Gram-positive and Gram-negative bacteria into the surrounding medium [30] and act on the host cells even when distanced from the site of infection.

1.1. Bacterial exotoxins

The bacterial exotoxins can be categorized according to different parameters, related with their function or main effects (e.g., enterotoxic, neurotoxic, hemolytic, cytotoxic, necrotic, lethal, etc). However, they are frequently pleiotropic and can influence different types of cells and tissues [30]. Since they show remarkable similarities in their mechanism of action, they are often characterized based upon on it [30]. Thus, knowing that some act at the cell surface and others at cytoplasmic targets, and according to their mode action, exotoxins can be classified into four groups [31] (figure 1):

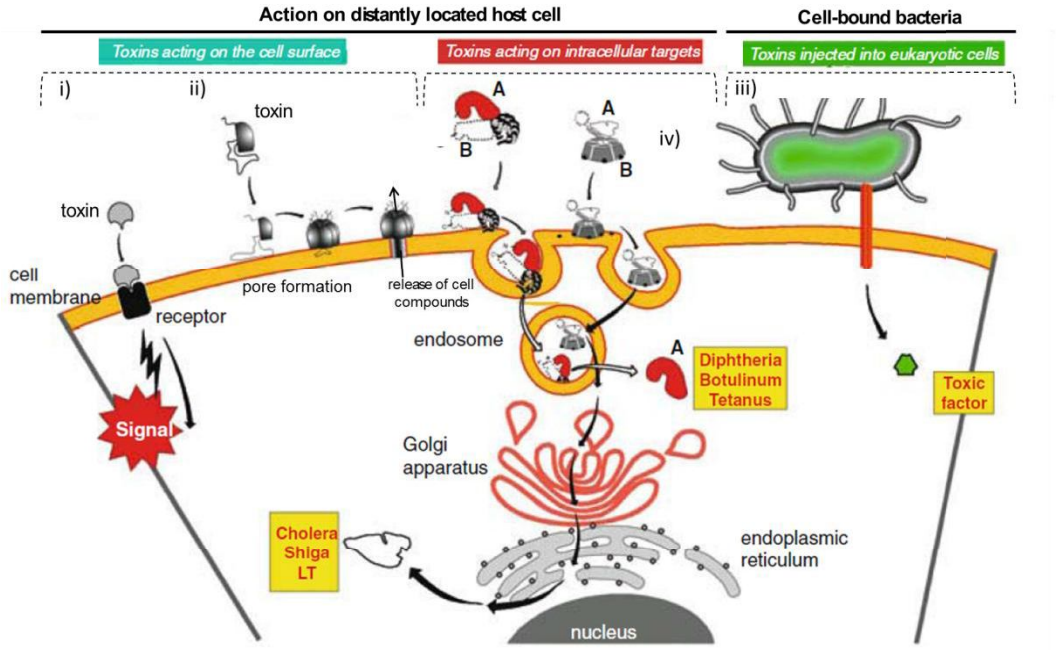

Figure 1. Schematic representation of the classes of bacterial protein toxins. There are four major classes of bacterial toxins, including: i) toxins that possess receptor-modulating activities, i.e., act by binding receptors on surface of host cell plasma membrane and trigger intracellular pathways; ii) toxins that bind to the host cell plasma membrane and disrupt the lipid membrane by pore formation or expression of phospholipase activity; iii) toxins that are injected directly from the bacterium into the cell by an injection apparatus that is a component of the bacterial pathogens (e.g., type III secretion system); iv) toxins that are internalized by receptor-mediated endocytosis, named AB toxins, where the A domain possesses enzymatic activity and the B domain is responsible for binding and translocation of the A domain into the cell cytosol. The toxin can be transferred directly from the endosomal compartment to the cytoplasm, driven by a pH-dependent conformation change, or can follow retrograde transport to the endoplasmic reticulum, from where they translocate to the cytosol. The group i) ii) and iv) often act at a site within the host that is distant from the bacterial pathogen, while the group iii) acts by contact with the host cell. (Adapted from [32])

i) Toxins that act at the cell surface by receptor-modulating activities

This class of toxins intoxicates by acting as host receptor agonists or antagonists, corrupting signal transduction pathways [31]. The activation or modification of secondary messengers, can cause dramatic alterations to signal transduction pathways critical in maintaining a variety of cellular functions [27]. They exert their action without directly killing the intoxicated cell. Examples of these related bacterial protein toxins are: i) heat-stable toxin a (STa), produced by a variety of enteric pathogenic organisms, which bind its cellular receptor, guanylate cyclase C (GC-C) receptor, by mimicking guanylin, a protein ligand of GC-C, resulting in the stimulation of membrane-bound guanylate cyclase that, in turn, leads to an increase in intracellular cyclic guanosine monophosphate (cGMP), affecting electrolyte flux in the bowel [27, 33]; or ii) superantigens, mostly produced by *Staphylocccus* and *Streptococcus*, which sequentially crosslink the major histocompatibility complex II (MHC-II) to T-cell receptors, expressed at the surface of antigen-presenting cells and T-cells, respectively. This leads to nonspecific activation of T-

cells, resulting in uncontrolled production of large and sudden amounts of cytokines causing fever, shock and death [31, 34-36].

ii) Toxins that act at the cell surface by damaging the plasma membrane

The action of toxins that mediate cellular damage lead to the disintegration of the cell membrane, which may result not only in the direct lysis of cells but it can also facilitate bacterial spread through tissues [27]. Toxins included in this group do so by different mechanisms: enzymatic degradation of the lipid membrane (e.g., hyaluronidases, collagenases and phospolipases) [27] or pore formation. In this last one, as its name suggests, bacteria release pore-forming toxins (PFT), which form stable multimeric structures of different size and molecular selectivity [31] that insert into the membrane (insertion as an α-helix to α-PFT or as β–sheet to β–PFT) [33], causing membrane permeability and ion imbalance [30, 33].

iii) Toxins that have an intracellular target and are directly delivered by bacteria into the cell cytosol

In this case, it is demanded cell to cell contact. After bacterial docking to the cell membrane, toxins are delivered into the cell cytosol by injectisomes, such as the type III secretion system, which consists in a multicomponent molecular 'syringe' that allows the secretion of effectors through the inner and outer bacterial membranes directly coupled to the translocation across the eukaryotic cell membrane [37]. It represents a more complex injection machinery of virulence factor directly from bacteria to cell [38], often paralyzing the host cell´s ability to neutralize the bacterial pathogen [31].

iv) Toxins that have an intracellular target and are internalized by receptor-mediated endocytosis

In contrast to the ones described above, delivery of this group of toxins into cells is independent of cell to cell contact, thereby acting locally and distantly of the site of infection. These toxins are frequently endowed with a major virulent function [31]. They are internalized by receptor-mediated endocytosis and reach their cytosolic target either by direct translocation from the endosomal compartment or from the endoplasmic reticulum after retrograde transport. The toxin focused in this work, an AB toxin, falls into this category, therefore a more detailed description is provided below.

1.2. AB Toxins

Many bacterial toxins achieve their high toxicity by delivering a catalytically active polypeptide fragment of the toxin to the cytosol of eukaryotic cells, attacking essential constituents [33]. Part of its great potency is due to high specific effects in the cytosol or in the nucleus, in most cases by enzymatic role, which leads to cell death or other effects on

cellular physiology in the host. In general, they act intracellularly as enzymes categorized in transferases, hydrolasases or lyases [29].

A wide range of intracellular targets are described for this type of toxins and some of their effects on cells can extend from inhibition of protein biosynthesis, increase in second messenger cyclic adenosine monophosphate (cAMP), disaggregation of the microfilament system to inhibition of neurotransmitter release [30] (table 1).

Table 1. Mode action of some AB toxins, their activity and cellular target. (Adapted from [39])

Toxin	Enzymatic Activity	Cellular Target(s)	References
Inhibit protein synthesis			
Diphtheria toxin	ADP-ribosyltranferase	Elongation factor 2 (EF-2)	[5]
Shiga toxin	N-glycosylase	28S rRNA	[15]
Pseudomonas exotoxina A	ADP-ribosyltransferase	EF-2	[12]
Active second messenger			
E.coli Heat-labile toxin	ADP-ribosyltransferase	G-proteins	[11]
Anthrax edema factor	Adenylate cyclase	cAMP-modulated protein	[4]
Clostridium botulinum C2 toxin	ADP-ribosyltransferase	Monomeric G-actin	[10]
Clostridium difficile toxins A and B	Glucosyltranferase	Rho G-proteins	[13]
Cholera toxin	ADP-ribosyltransferase	G-proteins	[7]
Pertussis toxin	ADP-ribosyltransferase	G-proteins	[14]
Protease			
Anthrax letal factor	Zinc endoprotease	MAPKK	[20]
Clostridial neurotoxins	Zinc endoprotease	VAMP/synaptobrevin, SNAP-25, syntaxin 1	[21]

Abbreviation:SNAP-25, synaptosomal- associated protein

The action in the cytoplasmatic targets, by these proteins, implies that they cross a biological membrane to reach the intracellular milieu. However, and excluding rare exceptions, such as *Bordetella pertussis* AC (adenylate cyclase) toxin [38], many of these toxins are not able to directly cross the plasma membrane. Its overall structural and functional architecture allow them to solve this, being these organized in a so-called AB-type structure [40].

Their cell entrance is via receptor-mediated endocytosis and the delivery of their enzymatic domain into the cytosol occurs either through the endocytic vesicle membrane or the endoplasmic reticulum membrane [25]. Their process of cell intoxication has been traditionally divided into four steps: binding to the host cell's receptor, internalization, translocation of the membrane and intracellular effect [39].

Thus, the intoxication process by toxins with intracellular targets, despite having different effects on the individual cell and on the organism as a whole, is characterized by a corresponding toxin structural organization in general [29].

1.2.1. AB toxin structure

AB toxins are characterized by a bifunctional AB structure, being physically organized into distinct domains. These possess a catalytic moiety (or A domain), usually with enzymatic activity responsible for inducing toxicity by modifying a cellular target upon entry into the cytosol, and a binding moiety (or B domain) that comprises the receptor-binding function, often providing tropism to specific cell types [33] and allowing the delivery of the catalytic domain into cells. In addition, the B promoter can also include a domain involved in membrane translocation (T) [29] that plays a role in the delivery of the A domain across a lipid bilayer [33].

Though distinct functions are performed by A and B subunits in the same infection process, the functional domains are localized either on distinct structures of a unique protein chain (single-chain toxin) or on several protein chains (multicomponent toxin) [38], the latter being produced separately by the bacterium (figure 2).

Single-chain toxins, related to diphtheria toxin (DT), Clostridial neurotoxins (tetanus neurotoxin, TeNT; and botulinum neurotoxin, BoNT) or *Pseudomonas exotoxin A* (PE), comprise the A and B domains contiguous (AB), in most cases, linked together via an exposed peptide loop and by an interchain disulfide bond [33].

In multicomponent toxins different scenarios are described. In addition to an A subunit single polypeptide, composed of two domains (A1 and A2) linked together via a disulfide bond, the toxin has a B domain that possesses a pentameric form (AB_5), and the assembly of both subunits occurs in the periplasmic compartment of the bacteria, during initial folding of the toxin [29]. A1 comprises the catalytic domain and A2, by penetrating into the central pore of the pentameric B subunit, allows non-covalently anchoring of A and B subunits [40]. This structure is shown for example in the cholera toxin (CT) or Shiga toxin (ST). Alternatively, multicomponent toxins may have multiple A subunits bound to the heptameric or octameric B domain ($AB_{7/8}$), such as in anthrax and *Clostridium botulinum* C2 toxin [33] that, in general, self-assemble during the process of intoxication, at the surface of the target cell [29]. The non-covalent interaction allows the association of theses multiple components and the formation of the final complex toxin [25]. Multicomponent toxins are also reported to have AB_2 and A_2B_5 structures, even though they only represent a minority of the toxins known so far.

The structure of the cytolethal distending toxin (CDT) was shown to be an AB_2 toxin [41-44]. The CDT holotoxin consists of three subunits: CdtA, CdtB and CdtC,

encoded by three genes organized in one operon [45]. The CdtA and CdtC subunits are required to mediate the binding on the surface of the target cells, allowing internalization of the active CdtB, which is functionally homologous to the mammalian deoxyribonuclease I [43].

Recently, the structure of the CDT-like typhoid toxin has been solved and shown to be an A_2B_5 toxin [46]. In typhoid toxin, similarly to CDT, the toxin is encoded by different genes organized in one operon. The CdtB gene is not associated with CdtA and CdtC, but to PltA and PltB, encoding, respectively, the pertussis-like toxin A (homologous to the pertussis toxin ADP-ribosyltransferase subunit) and the pertussis-like toxin B (homologous to one of the pertussis B subunits) [43]. The 2A domain comprises one unit of CdtB and one unit of PltA and the B5 domain comprises five PltB subunits. CdtB and PltA are joined by a disulphide bond that is formed by two unique cysteine residues that are absent in other cytolethal distending toxins [47].

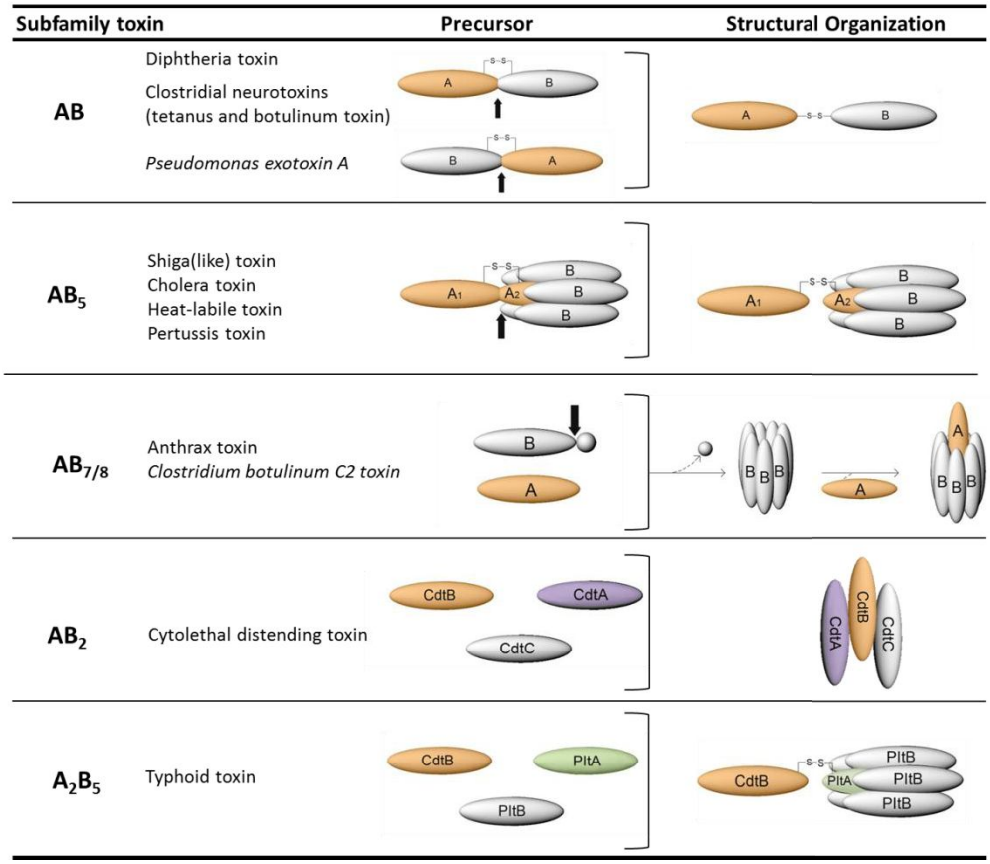

Figure 2. Structural organization of AB toxins subfamily. AB toxins are composed by a catalytic domain, the A moiety (orange and/or green), and a binding domain, the B moiety (gray and/or purple). They are produced by bacteria as a single-polypeptide precursor or as a multicomponent toxin. In the multicomponent toxin form different ways are shown: the toxin is composed by a single A subunit, subdivided in two (A1 and A2), linked via a disulfide bond, and a B domain pentameric form (AB_5), occurring the assembly of both subunits in the perisplasmic compartment of the bacteria; or by multiple linked A subunits and a heptameric or octameric formed B ($AB_{7/8}$), occurring the self-assemble of the toxin, outside the host cell. The proteolytic cleavage (indicated by closed arrows) is required to assemble the toxin ($AB_{7/8}$) or to convert a

precursor molecule into an active one (AB, AB_5), usually between two cysteine residues. Multicomponent toxins are also reported to have AB2 and A2B5 structures.

Thus, the structure featured in AB toxins is, in part, influenced by the composition of B components, which allow their subdivision into subfamilies (AB, AB_5, $AB_{7/8}$, AB_2 and A_2B_5,) and contributes to a variety of ways to arrange them (figure 2). In cellular uptake, to reach the cytosolic target, these toxins do so via different modes of binding that correspond to different structural organizations [29].

Given that, commonly, an AB-toxin is synthesized in an inactive form, it is required proteolytic processing. On the one hand, this may simply lead to the conversion of the precursor molecule to an active one, as happens to some single chain toxins, or to the complex toxins previously assembled by the bacteria, e.g. CT and ST; on the other hand, this processing may be required to the assembly of the toxin, such as in anthrax and *Clostridium botulinum C2*, given that the cleavage of the B moiety exposes a site that then binds to the A moiety non-covalently [33].

The proteolytic cleavage of the toxin can occur by a protease from the producing organism (e.g. CT or Clostridial neurotoxins) or by host proteases (often furin) [33], normally within the linker region flanked by two cysteine residues (figure 2). The evidence implicating the furin protease in the activation of toxins emerged in a systematic study of the activation of anthrax toxin protective antigen (PA) [48]. Rapidly was understood that in other toxins such as ST, DT and PE, processing is also performed by this protease [39]. However, for this last one, and exceptionally, it is only carried out after toxin internalization. In case of DT, both processing at the cell surface mediated by furin or furin-like proteases as action of bacterial proteases are described [49, 50].

Once the toxin is processed, the intoxication process will proceed. This process is characterized for having similar mechanisms of action, including four main steps until reaching the intracelular milieu: i) the binding, ii) internalization iii) translocation of the membrane and iv) intracellular effect [30].

1.2.2. Endocytosis and intracellular pathways of AB toxins

1.2.2.1. Binding and internalization of AB toxins

The initial step of intoxication by AB toxins relies on the specific interaction of the toxin's B subunit with a receptor at the cell surface. A large variety of compounds can be used as toxin receptors, including numerous lipids or lipid derivatives (glycolipid, gangliosides) and transmembrane proteins or glycoproteins [51]. The nature of the receptor and its localization, particularly in membrane lipid structures, such as lipid rafts, seem to be determinant in driving the intracellular trafficking of toxins and consequently in the targeting of the toxin to the organelle before it translocate to the cytosol [51]. Apart

exception, receptor specificity is critical for the pathogenic process, as it determinates host susceptibility, tissue tropism, and the nature and spectrum of the resultant pathology [40].

Once toxins bond to their respective receptors, they are embedded in endocytic vesicles generated by invagination of the cell membrane [51] and are internalized into the host cell via different pathways. Interestingly, the multiple combinations of endocytic processes and intracellular transportations discovered by toxins have proven to be valuable in understanding cell biology, in particular, endoplasmic reticulum (ER) and Golgi apparatus as well as endosomes [52] .

Most toxins are endocytosed, although by different mechanisms, before translocation to the cytosol. Some of these multiple ways of internalization include the well characterized clathrin-dependent and -independent pathways as well as caveolae/raft and dynamin-independent pathways [51]. As one might expect from opportunistic ligands such as toxins, usually one can enter the cell by more than one pathway [52]. However, the entry via clathrin-coated pits for DT, anthrax toxin, STis the preferential route [25]. In contrast, the CT is endocytosed via various mechanisms involving clathrin- and caveolin-dependent and independent mechanism [25, 53].

Once into endosomes, different intracellular routes can be carried. Given that most of the AB toxins described belong to AB, AB_5 or $AB_{7/8}$, we will consider only the pathways followed by these toxin types.

1.1.2.2. Translocation and/or intracellular trafficking routes

Despite the diversity of the entry requirements and routes, most AB toxins interact at same point with early endosomes, and very few reach later stages of the endocytic pathway [25]. For some, mainly AB single chain and free multi-component toxins ($AB_{7/8}$), apart exception, this is the final destination before translocation of the catalytic domain. In contrast, assembled multicomponent toxins (AB_5) are only in transit in early or late endosomes from where they are directly routed to the trans-Golgi network, prior to delivery of their catalytic subunit [25]. Therefore, two main intracellular trafficking routes are used by these toxins, named i) short and ii) long pathways [38], respectively. In each pathway, the mechanism used by different toxins in the translocation of their catalytic domain or component is different and based on its structure (figure 3).

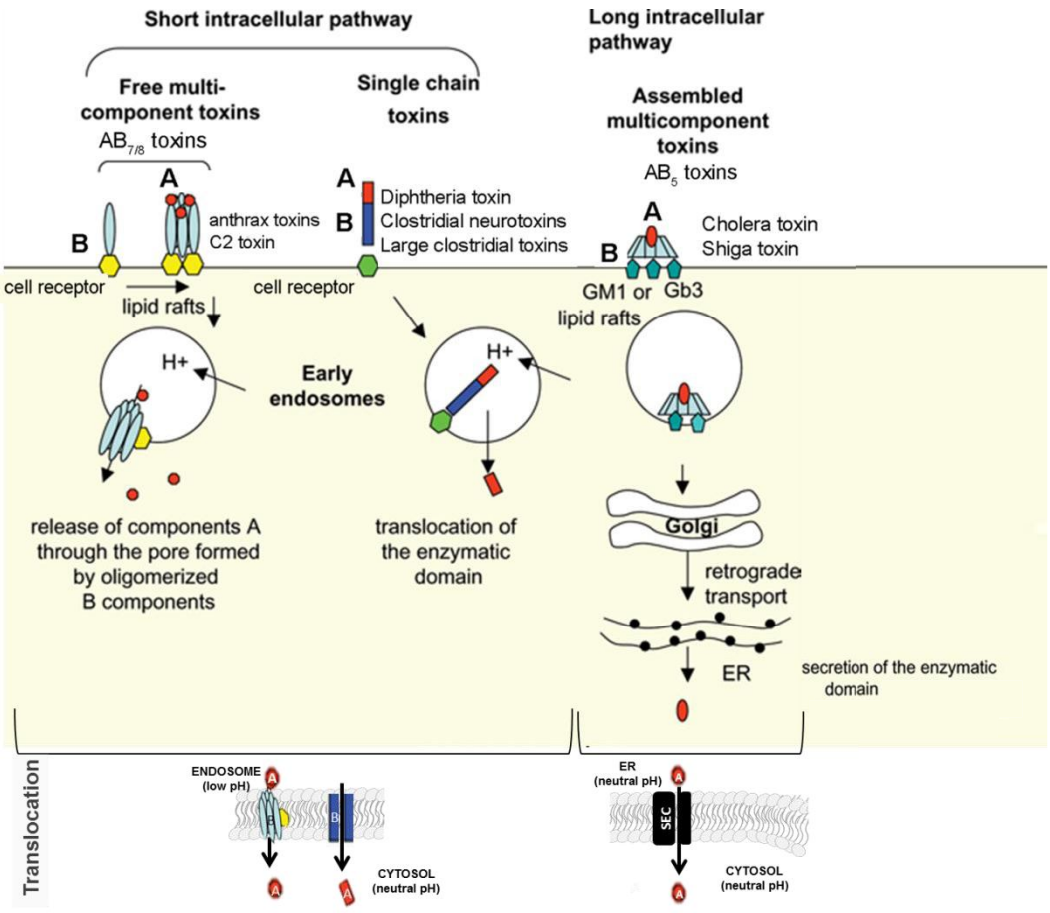

Figure 3. Schematic representation of the intracellular routes followed by different types of AB subfamilies. **Two main intracellular trafficking routes are used by these toxins named i) short or ii) long pathways, which differ in their final destination prior to delivery of their catalytic subunit (early endosome and RE, respectively) and the translocation mechanism of the catalytic domain into the cytosol.** The short pathway is followed by free multicomponent chains (AB7/8), e.g. Anthrax toxin and C.botulinum C2, or single -chain toxin (AB), including DT, Clostridial neurotoxins and large clostridial toxins, being characterized by pH gradient that induce a conformational change and membrane penetration of these toxins leading to its translocation. After binding to its receptor, the toxin is internalized by endocytosis. The endosomal acidic pH triggers a conformational change of the B subunit allowing its insertion into the membrane and the formation of ion-conducting channels across this membrane through which the catalytic subunit translocates into the cytosol. In the long pathway, used by assembled multicomponent toxins (AB5), e.g. CT and ST, the route to this release extends up to the ER, where the translocation, not induced by the pH gradient, occurs via ER secretion machinery. After binding to its receptor, localized in raft microdomains, the toxin is internalized into endocytic vesicles from where it enters to the trans Golgi network for subsequent transport to the endoplasmic reticulum where retrotranslocation of the enzymatic A sbunits occurs.

Abbreviators: GM1:Ganglioside; Gb3: glycosphingolipid. (Adapetd from [38])

1.1.2.2.1. Short trafficking pathway and translocation

Both single-chain toxin, such as DT, BoNT and TeNT, and free multi-component toxins, including anthrax or C. *botulinum* C2 toxin, use this route to reach their target. After binding and internalization by endocytosis into the cell, these toxins migrate into the early or late endosomes, from where translocation of the enzymatic domain into the cytosol occurs upon a pH gradient [38]. Acidification of the vesicles is a prerequisite step for translocation, given that it triggers a proteolytic processing and/or conformational change

of the toxin, allowing the delivery of its catalytic domain through the lipid membrane of this compartments [38]. Thus, transmembrane pH gradient provides the driving force for the translocation [29].

The different mechanism used to deliver the A subunit by the single chain toxins or the B oligomeric toxins, across lipid membranes, lead these toxins to be treated separately.

a) Single-chain toxin (AB): diphtheria toxin as an example.

The best-characterized paradigm of a single-chain toxin translocation is that taken by DT, secreted by *Corynebacterium diphtheria* [38]. The bond to the receptor is done by its C-terminal domain (R), carried by endocytosis into the clathrin coated vesicles and transported to early–late endosomes. The acidic endosomal pH (pH<6) [37] triggers a conformational change of the B subunit, facilitating the insertion of α-helices TH5–TH7 and TH8–TH9 of the translocation domain (T) into the membrane, forming a cation selective pore [38, 54]. In addition, it also leads to a conformational change in the A chain, becoming partly unfolded and hydrophobic, which allows its insertion into the endosomal lipid membrane and subsequently its translocation to the cytosol via the formed channel [29]. The interchain disulfide bridge between A and T domains is reduced during the translocation process, allowing the catalytic subunit to leave behind the protomer B ion channel and to be refolded in the neutral pH of the cytosol. It is known that chaperones, together with other cytosolic factors, are also required for the translocation and refolding of the A domain [37]. Once in the cytoplasm, the A subunit catalysis the transfer of the ADP-ribose of NAD to the elongation factor 2 (EF-2) leading to the inhibition of protein synthesis [55].

b) Free multi-component toxins ($AB_{7/8}$): anthrax toxin as an example

In contrast, in free multi-component toxins ($AB_{7/8}$) the translocation mechanism of the enzymatic component is based on pore formation through the membrane of endosomal vesicles. At low pH, B components (binding domains) of these toxins oligomerize and insert into vesicle's lipid membrane [37, 51]. This leads to the formation of a channel, similar to the β-PFTs, that allows the release of the catalytic domain A into the cytosol. This mechanism of translocation contrasts with the one above described, where the B subunit plays an active role in the insertion of the catalytic subunit and the toxin interacts with the lipid membranes both by the translocation and the enzymatic domains [51].

Since in these toxins, the enzymatic and binding components are distinct constituents, each type of protein can be assembled separately, forming structures appropriate for an efficient translocation [39]. For toxins in this group, the anthrax toxin is the best characterized [51].

The anthrax enterotoxin, produced by *Bacillus anthracis*, unlike AB toxins described above, has a tripartite structure, consisting of three independent polypeptide chains: a cellular binding moiety, protective antigen (PA), and two catalytic moieties of the toxins, lethal factor (LF) and edema factor (EF) [56]. During infection, PA (PA_{83}) binds to the cell surface receptors, capillary morphogenesis protein 2 (CMG2) or tumor endothelium marker 8 (TEM8) receptors, and its proteolytically processing by furin or furin-like proteases result in the formation of a 63 kDa C-terminal fragment (PA_{63}) and the liberation of a N-terminal fragment (PA_{20}) [37, 56]. The fragment PA_{63} then spontaneously forms a heptamer or octamer ring-shaped structure often referred to as the pre-pore [57]. It is now known that the PA can also be processed in circulation by unidentified proteases, resulting in PA_{63} [55]. In both cases, the pathway that follows is the same, as described below. The PA_{63} oligomer–receptor complex is capable of binding three EF and/or four LF molecules and then is internalized through a receptor-mediated endocytic pathway, via clathrin coated pits [58]. Once in the endosomes, acidic conditions induce the conversion of the PA_{63} oligomer pre-pore in to a protein-conducting channel (pore) in the membrane, through which LF and EF are translocated into the cytosol to exert their cytotoxic effects: the metalloprotease LF cleaves MAP kinases kinases, inhibiting it, and the EF increases the cAMP level [55].

1.1.2.2.2. Long trafficking pathway and translocation

In some toxins such as assembled multicomponent toxins (AB_5), the delivery of the catalytic domain into the host cell happens after the retrograde transport through the Golgi apparatus and the ER [51]. This is designated long pathway and it is related to the CT and ST. In contrast to the short pathway, in which translocation of the catalytic domain occurs in early-late endosomes, in the long pathway, the route to this release extends up to the ER.[51]. In addition, the pH gradient does not appear to induce a conformational change and membrane penetration for these toxins [29].

a) Assembled multicomponent toxins (AB_5): Cholera toxin as an example

Here, the CT is taken as an example of a toxin that uses this delivery route. It is an oligomeric protein produced by *Vibrio cholera*, composed by an A subunit that, although being synthesized as a single polypeptide chain, is post-translationally modified through the action of a *V. cholerae* protease generating two fragments, CTA1 and CTA2, which however still remain linked by a disulphide bond [59]. These two fragments are bond with a ring-shaped pentamer formed by B-subunits. The assembly of the toxin results in a hexameric protein complex and occurs in the bacterial periplasm [53]. Its binding to the ganglioside (GM1) receptor, localized at the epithelial cell surface in raft microdomains [51], allows it to internalize via different pathways, such as lipid raft/caveolae mediated

endocytic pathway, clathrin mediated endocytic pathway or ADP-ribosylation factor 6 (Arf6)-associated endocytic pathway [60] to the Golgi via early and recycling endosomes. In the perinuclear region of the Golgi, the toxin enters to the endoplasmic reticulum and the catalytic domain is released from the rest of the toxin and transferred into the cytosol, through the Sec61 channel, in an unfolded state. Once in the cytosol, it is folded and the CT enzymatic domain binds to NAD and catalyzes the ADP ribosylation of GTP-binding protein G of the adenylate cyclase complex, increasing intracellular cAMP [60].

Despite these examples, other alternative intracellular pathways are reported.

In the last decades, these discoveries have been proving that, although the toxins have been very helpful in detecting and clarifying the role of different molecules, such as G proteins [27], they are also a powerful tools to reveal new pathways in intracellular transport, namely, types of endocytosis, membrane transport to various intracellular destinations such as the Golgi apparatus and the ER as well as to late endosomes/lysosomes or translocation to the cytosol from endosome, for example, by DT [27]. In fact, revelation that molecules can be transported from the cell surface through the Golgi apparatus and to the ER has been made by studies of ST [61].

Since the discovery of a diversity of bacterial toxins, its properties and molecular mechanisms of toxin action are not only a relevant topic of study for those interested in the pathogenesis and immunology of infectious diseases but also they have been used as an instrument for gaining insights of fundamental processes of cell biology [62], allowing remarkable advances both in basic and applied sciences. Furthermore, knowledge about their action on cells can be used to combat infectious diseases where such toxins are involved and a whole new field of research takes advantage of their ability to enter the cytosol, given its structural characteristic and functional architecture, for therapeutic purposes in connection with a variety of diseases [27].

1.3. Toxins as pharmacological tool

In a pharmacological context, several properties characterize toxins and make them unique and interesting tools to be exploited in an attempt to combat numerous diseases, such as cancer or inflammatory diseases. The ability of acting on target cells independently of the presence of the producing bacteria [27], is one of them.

Some advantages of toxins as biotechnological tools come from their high potency, specificity and extraordinary efficiency [63]. They act at extremely low concentrations, in the range of the picogram, like in the case of botulinum neurotoxins, which grants its high specificity [63].This is achieved, on one hand, by the enzymatic action that most of toxins possess, not requiring other molecules to modify the target and, on the other hand, by the ability to interact with high-specific cell surface receptors in certain cells [62]. In addition,

in most cases, the fact that its catalytic mechanisms are irreversible and these toxins have preference to eukaryotic targets that play pivotal roles in cellular functions, make them high efficiency tools.

Some years ago, bearing in mind the properties mentioned earlier as well as the discovery of the ability to replace domains in intracellular target toxins, depending of the purpose of their use, led to the thought of AB toxins as potential biomedical, pharmaceutical and biotechnological tools.

1.3.1. AB toxins as biotechnology tool

In the last decades, the resolution of the structural properties of AB toxins allowed a major breakthrough providing significant structural insights into the biological, functional and catalytic activity of these toxins. The discovery that these toxins are often organized into discreet domains that comprises a catalytic, receptor binding and translocation domain allowed to development them as biotechnological tools. In other words, these toxins (or parts of them) can be used as vectors to bring other molecules into cells, a toxins' ability that is of great importance in medical and pharmaceutical areas [64].

Taking into account the main aim, all these three components can be used to construct a protein delivery system [27]. One of the properties exploited in these toxins is the possibility to transfer different molecules into cell, as enzymes, because they are able to cross the lipid cell membrane without major damage of cell integrity unlike lots of synthetic chemicals used as pharmacological tools [27]. Despite of intensive research in this area, the understanding of many of these mechanisms of toxin translocation, undeniably fascinating, remains a challenge [2].

Other property is the possibility of changing of the receptor-binding domain, which often provides tropism to specific cell types. This particularity takes advantage in cases of cells with membrane signing, as often happens in cancer cells. In addition, as mentioned above, it is known that these proteins attack targets of the cell that compromise its functions. That being said, these properties make these toxins particularly interesting in treatment of diseases like cancer, whose purpose is to kill cells. The concept of construction bimolecular agents chemically linked or genetically fused to a toxin moiety is already being explored for some years [62].

In the late 1970s, the discovery by Yamaizumi *et al*. of the potency of DT for mammalian cells [63], the introduction of the concept of using antibodies to redirect toxin killing activity in a purposeful way by Thorpe *et al*. [64], and the strategy to link both were milestones in the born of the “early” immunotoxins [65].

Immunotoxins (IT), chimeric proteins consisting of a targeting moiety linked to a toxin, explore the phenomenon where, as in cancer, cells overexpress several tumor

associated antigens, membrane receptors and carbohydrate antigens thus allowing death of diseased cells while leaving normal cells [65]. In immunotoxin constructs, the target moiety varies depending of the disease and tissue type [65]. They belong to one of two groups: (i) monoclonal antibody or single chain variable fragment (scFv) specific for the overexpressed tumor-associated antigen or membrane receptor; or (ii) natural ligand for the membrane receptor like cytokine, growth factor or peptide hormone [62] (figure 4). In this last category, Melanocyte-stimulating hormone (MSH) as alpha-MSH targeted to melanotropin receptors (MTR1) in melanoma [66] and IL-2 were early candidates [64] to link to DT. Although many other toxin-based cell delivery systems are under studies for this purpose, PE, anthrax toxin and DT are among the top choices for immunotoxin development [64].

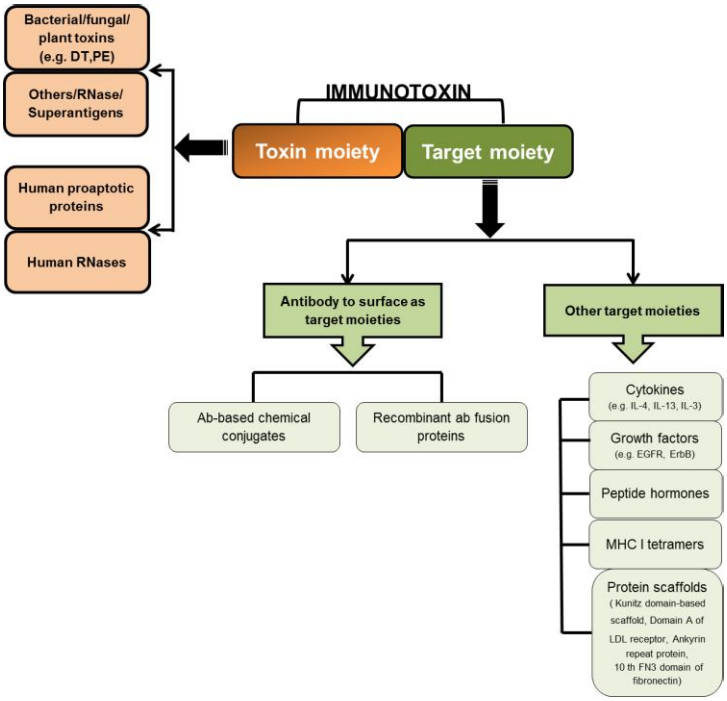

Figure 4. Schematic representation of various strategies for construction of immunotoxins. Immunotoxin (IT) is a chimeric protein consisting of a targeting moiety linked to a toxin. The targeting moiety belongs to one of the two groups: antibody to surface or others including cytokines or growth factor. (Adapted from [64])

Mostly, but not exclusively, immunotoxins are purpose-built to kill cancer cells as part of novel treatment approaches. However, other applications for immunotoxins include immune regulation and the treatment of viral or parasitic diseases [62].

Although to date only one targeted toxin, DT-IL2 (termed denileukin diftitox - trade name Ontak), directed to the IL2 receptor, has been approved for humans (FDA approved for cutaneous T cell lymphoma (CTCL) therapy) [62], other immunotoxins await the approval of favorable results from phase III trials [65]. Numerous studies to various diseases are being developed and have been successful. If we examine on-going clinical

trials, we see these kinds of efforts persist. In many cases, the toxin constructs that selectively target cells are already in phase I, II or III clinic trial [63, 65] (table 2). This is exemplified by DT where the receptor binding domain was exchanged with interleukin-3 (IL3). This is a cytokine that supports proliferation and differentiation of multipotential and committed myeloid and lymphoid progenitors [67]. Myeloid leukemic progenitors over-express IL3R, which is composed by two units: α and β; in contrast, normal mature myeloid cells lack IL3R [65]. The binding of IL3 to its receptor causes rapid receptor-mediated endocytosis on receptor coupling [68]. These characteristics were harnessed and IL3 has been engineered as receptor binding component of the immunotoxin. DT-IL3 has been utilized as a potent toxin targeting Acute Myeloid Leukemia (AML) and it already in phase I clinical trial [69]. Other example in phase I clinical trial is ErbB2 (Her2), a growth factor receptor generally overexpressed in solid tumors, that is targeted by using single chain fragment variable (scFV(FRP5))-PE for breast, prostate, head and neck and non-small cell lung cancer [63].

Table 2. Clinically evaluated/under evaluation of immunotoxins (AB toxins) used to solid tumors, hematologic malignancies and autoimmune disorders. (Adapted from [64]

Target	Targeting moiety	Toxin	Target tissues/diseases	Experimental phase	Reference
Solid tumors					
IL4R	IL4	PE	Glioblastoma, astrocytoma, head and neck squamous carcionama, prostate carcinoma, lymphoma, kydney, lung and breast cancer	I/II	[3]
IL13R	IL13	PE	Glioma, renal cell carcinoma, ovarian cancer, ks, head and neck carcinoma	I/II/III	[6]
IL2R	IL2	DT	melanoma, ovarian, breast, kidey cancers, KS	II	[18]
ErbB2(Her2)	dsFV	PE	Ovarian, squamous cell, prostate, head and neck, NSCLC	I	[23]
VEGFR	VEGFR	PE	Glioma, renal cell carcinoma, ovarian cancer, ks, head and neck carcinoma	In vivo	[22]
TfR	Transferrin	DT	Brain and CNS tumors	I/II	[19]
Leukemia and lymphoma					
IL2 R	IL-2	DT	B-cel and t-cell leukemia and lympomas	I, II, III, IV FDA approved for CTCL therapy	[2]
IL2 α R(CD25)	ScFv	PE	Leukemia and lymphoma	II	[8]
IL3 R	IL3 variant	DT	AML, MDS	I	[17]
CD64	Mab	PE	AML	In vivo	[9]
Autoimmune disorders					
IL2-R	IL2	DT	GVHD, psoriasis, RA	I/II	[1]
FR β	Mab	PE	RA	In vivo	[16]

Abbreviations: IL(R): Interleukin (receptor); TNFR: tumor necrocis factor receptor; EGFR: epidermal growth factor receptor; VEGF: vascular endothelial growth factor; TfR: transferrin receptor; DT: Diphteria toxin; Pseudomanas exotoxin (PE); monoclonal antibody (Mab); disulfide-stabilized Fv antibody fragment (dsFv); scFv: single chain variable fragment; NSCLC: non-small cell lung cancer; AML: acute myelogenous leukemia; FR β: Folate receptor β; RA: Rheumatoid arthritis; GvHD: Graft versus Host Disease

The advances and successes in immunotoxins are the result of extensive research and cooperation between different scientific areas. A better knowledge of the toxins described some years ago, such as the boundaries of each domain as well the role played by specific amino acids regions in their functions, have allowed an undeniable breakthrough in the development of immunotoxins. For example, we already are in the

presence of the third generation of immunotoxins, where improvements of molecules, like the removal of the immunogenic amino acids, are being made [65]. In fact, in spite of the potential shown by bacterial toxin-based chimeric proteins, the immunogenicity has been one of the several obstacles that has limited their clinical application [62]. However, the improvement of immunotoxin design, as described above, has been trying to minimize these side effects.

Further, the discovery of new AB toxins with different target moieties of the ones we know so far, such as nuclear factor-κB (NF-κB), which play a crucial role in several diseases, have also opened doors in the search to treatment for them. However, characterization and understanding of its functions in anticipation to the scientific challenges and strategic priorities to use as biotechnology tool are required. An example of these new AB toxins is AIP56.

2. AIP56

2.1. AIP56-associated pathogenecity

AIP56 (**A**poptosis **I**nducing **P**rotein of 56 kD) is a plasmid-encoded exotoxin, abundantly secreted by virulent strains of *Photobacterium damselae* ssp *piscicida* (*Phdp*), a gram-negative pathogen that infects and causes high mortality in several warm water fish species worldwide, including sea bass [70]. The toxin induces selective apoptosis of fish macrophages and neutrophils, resulting in extensive lysis of the phagocytes by post-apoptotic secondary necrosis[71]. The fails in the removal of the apoptotic cells and apoptotic bodies, i.e. cellular condensation induced by the apoptotic process, lead the transition of the apoptotic process to secondary necrosis and subsequently cell lysis, which is pathogenetic [72, 73].

During infection, *Phdp* spreads through the bloodstream, colonizes various internal organs and secretes the AIP56 toxin [73]. Apoptosing cells, identified by an apoptotic morphotype, TUNEL positivity and presence of active caspase-3, are seen in foci and scattered in several organs including kidney, spleen and liver, which exhibit hemorrhagic septicaemia and granulomatous lesions [73]. Similar pathological signs are observed when AIP56 is inoculated alone into fish, showing that the toxin is the key virulence factor responsible for the *Phdp*-associated pathology [63, 64].

2.2. AIP56 structure and action

AIP56 is classified as a single-chain AB-type toxin [74]. Analysis of its primary structure showed that AIP56 is synthesized as a 520 amino acid precursor protein, including an N-terminal signal peptide that is cleaved during secretion, originating a 497-

amino acid mature toxin [70]. An important features present in the N-terminal region is a zinc-binding signature HExxH, typicall of most zinc metallopeptidases [75], consisting of the amino acid sequence HEIVH [70], similarly to the tetanus neurotoxin [76]. An additional feature is the presence of only two cysteine residues (C^{262} and C^{298}) [70].

Homology analysis of AIP56 showed similarity with other proteins from different organisms [73] (figure 5), especially full-length uncharacterized homologues in *Vibrio* species but also *Arsenophonus nasoniae*. Furthermore, the N-terminal region (first 324 amino acids) was found to be homologous to the non-LEE (locus of enterocyte effacement) encoded type III secreted effector C (NleC), conserved in several enteric pathogenic bacteria, while the C-terminal region share homology to an uncharacterized protein of *Acrythosiphon pisum* bacteriophage APSE-2 and to the C-terminal portion of a hypothetical protein of the monarch butterfly *Danaus plexippus* [73]. These facts, suggesting that AIP56 was a two-domain protein, were later confirmed by limited proteolysis [73].

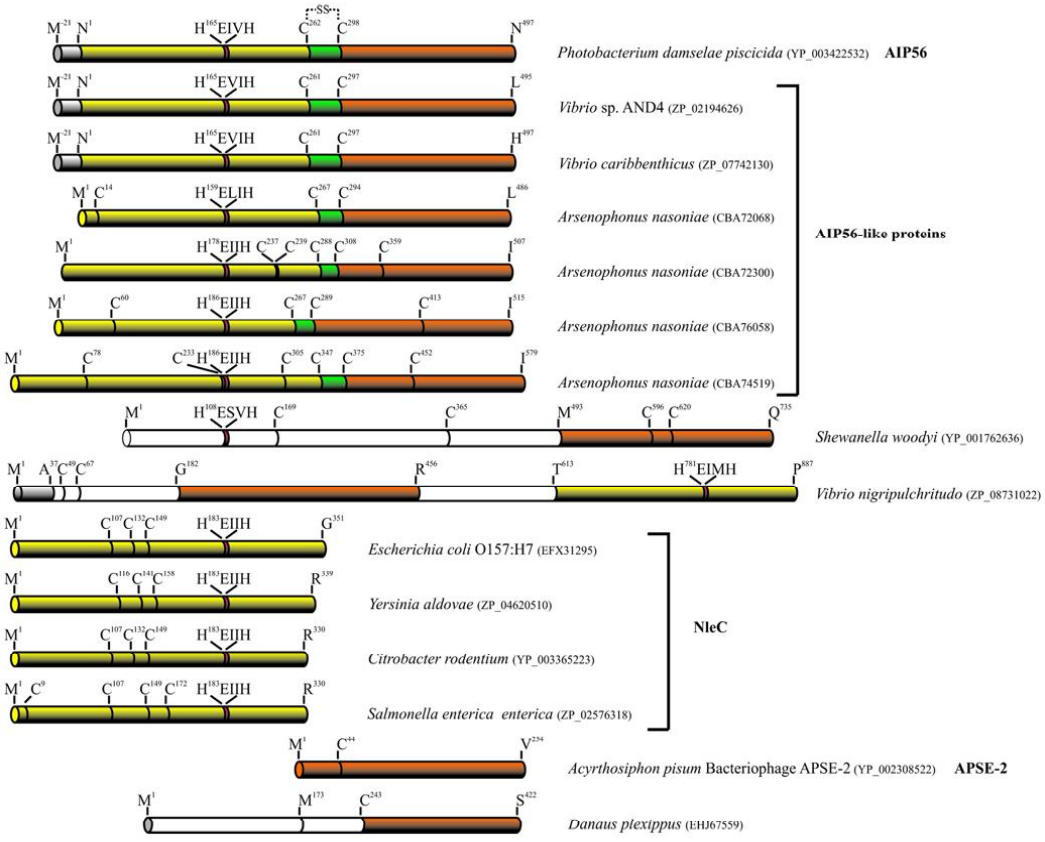

Figure 5. Schematic diagram of the AIP56´s primary structure and AIP56-related proteins. Grey: signal peptide, as experimentally determined for AIP56 [70]; Yellow: regions with high identity to NleC and AIP56 N-terminal catalytic domain; Green: regions with high identity with AIP56 linker polypeptide; Orange: regions with high identity to APSE-2 and AIP56 C-terminal domain; Red: zinc-metalloprotease signature HEXXH; White: regions with low identity to AIP56 domains, NleC or APSE-2. Conserved zinc-metalloprotease signature HEXXH, cysteine residues and other signaled amino acids are represented and numbered at their relative positions. Information about AIP56-related proteins is constantly being updated. Examples includes Vibrio azureus (WP_021710670.1) and Vibrio tasmaniensis (WP_017104811.1) (From [74])

As a single-chain toxin, AIP56 is organized in two distinct domains (figure 6): an N-terminal A domain, which displays zinc-metalloprotease activity by cleaving NF-κB p65 at the Cys^{39}(Cys^{38} of human p65)-Glu^{40} peptide bond within the p65 N-terminal Rel homology domain (segment crucial for DNA interaction), and a C-terminal B domain, involved in the binding and internalization of the toxin into the host cells [74]. These two domains are connected by a disulphide bridge, formed by C^{268} and C^{298}, which plays a role in the intoxication process but, apparently, may not be an absolute requirement for toxicity. In addition, the integrity of the linker region between the two cysteine residues is needed for toxin internalization [74]. AIP56-mediated depletion of NF-κB p65 likely explains the disseminated phagocyte apoptosis observed in *Phdp* infections, which contributes to subvert the host immune response and determines the outcome of the infection [70, 74, 77].

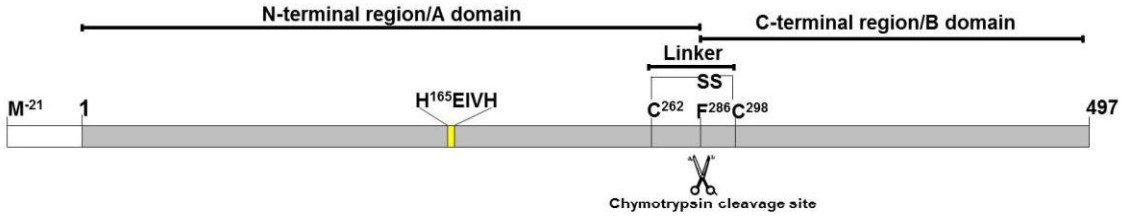

Figure 6. Schematic representation of AIP56. The 497-amino acid mature toxin (gray) has two domains: an A domain and a B domain linked by a disulphide bridge between cysteine 262 and 298. The conserved zinc-metalloprotease signature (HExxH) is represented and numbered at its relative positions. The cleavage site resulting from limited proteolysis by chymotrypsin is depicted. (Adapted from [74])

Interestingly, both AIP56 and Nlec were found to have a similar proteolytic activity towards p65 within its N-terminal region, by cleavage of this subunit at the same peptide bond [74, 78]. Contrasting are the requirements for their activity since NleC requires a type III secretion machinery, while AIP56 has an intrinsic ability to reach the cytosolic target owing to its structure-function, namely a C-terminal B domain responsible by its delivery into the cell [74].

As an AB toxin, AIP56 enters cells through a multi-step process. Analogously to other AB toxins, such as DT and *Clostridial* neurotoxins, it is known that AIP56 is endocytosed by a clathrin-dependent mechanism and, triggered by the acidic endosomal pH, suffers conformational changes, allowing its translocation into the cytosol (in press). Nevertheless, the detailed molecular mechanisms leading to these observations are still unknown. Efforts to obtain a better knowledge about those mechanisms as well as better structural-function characterization of the toxin, namely, the minimal region involved in translocation and receptor-binding and the boundary between A and B domains, are presently ongoing. Yet, recent studies showed that, similarly to fish macrophages and neutrophils, AIP56 is also able to induce depletion of NF-κB p65 and apoptosis in mouse

bone marrow derived macrophages (mBMDM) (in press).These results, in addition to its structural arrangement and how cell intoxication occurs, confers to AIP56 an enormous biotechnological potential and have relevant implications when considering its use as a therapeutic agent in situations associated with uncontrolled activation of NF-κB [73], such as inflammatory diseases and cancer.

The target of this toxin is an important feature, since that the transcription factor NF-κB is also involved in several crucial cellular processes, described in greater detail below.

3. NF-κB

For over a quarter-century ago, Sen and Baltimore [79] firstly described the transcription factor NF-κB (nuclear factor-kB) as a protein that bounds to a specific decameric DNA-sequence (5'-GGGACTTTCC-3') within the intronic enhancer of the immunoglobulin kappa light chain gene in mature B and plasma cells but not pre-B cells [80]. An intense research followed and soon afterwards it became clear that this consisted of a family of transcription factors, which includes a collection of proteins, conserved from (at least) the *phylum Cnidaria* to humans [81], that are expressed in almost all cell types and regulate many target genes with a whole variety of functions [82].

During the past two decades, several studies have highlighted its most important and conserved evolutionarily role in immune, inflammatory and stress responses through NF-κB-dependent transcription of cytokines, chemokines, cell adhesion molecules, growth factors, factors of the complement cascade, acute phase proteins, effector enzymes in response to ligation of many receptors involved in the immune recognition process, including T-cell receptors (TCRs) and B-cell receptors (BCRs) for example [83-85]. This way, it allows cells to adapt and respond to environmental changes, a process pivotal for survival [86]. Even though its key role, it is well-established that it acts broadly, being able to regulate the expression of genes outside them, influencing events that impact proliferation, differentiation, cell survival, apoptosis and development of a number of tissues including central nervous system, skin, mammary gland and bone or embryonic development [83].

So far, it is known that several external stimuli, which are continually expanding, can lead to its activation such as bacterial and viral infections (e.g., through recognition of microbial products by receptors such as the Toll-like receptors), inflammatory cytokines, antigen receptor engagement, upon physical (UV- or γ-irradiation), physiological (ischemia and hyperosmotic shock) to oxidative stresses [86, 87].

Taken into account its critical play in mediating responses to a remarkable diversity of external stimuli and the wide role on cell physiology it is not surprising that

dysregulation of NF-κB is linked to multiple physiological and pathological processes such as inflammatory and autoimmune diseases as well as cancer [86].

3.1. The NF-κB transcription factor family

In mammals, the NF-κB proteins family is composed of p50 (NF-κB1) and p52 (NF-κB2), synthesized as pro-forms (p105 and p100, respectively) that undergo proteolytic process to become shorter and active DNA-binding proteins; and c-Rel, RelB and RelA (p65) [81]. The five members of this protein family share some structural features, including a conserved 300 amino acid long amino-terminal Rel homology domain (RHD), which is essential for dimerization, binding to cognate DNA elements and interaction with IkBs [82]. Structurally, the RHD is divided into three functional subdomains: the N-terminal domain (NTD), the dimerization domain (DiD) and the nuclear localization signal (NLS) [88]. In contrast, and based on their transactivation potential, the C-terminal these proteins are distinguishable. c-Rel, RelB, and p65 proteins contain C-terminal transactivation domains (TAD), necessary for transcriptional activation, often not conserved at sequence level across species [81]. In opposition, p50 and p52 proteins lack TAD and instead contain a glycine-rich region (GRR), reminiscent of the larger precursors processing p105 and p100, respectively [89] (figure 7). As consequence, dimers of p50 and p52, which bind to NF-κB elements of gene promoters, act as transcriptional repressors. However, when bound to members containing a TAD, such as p65 or RelB, they constitute a transcriptional activator [82].

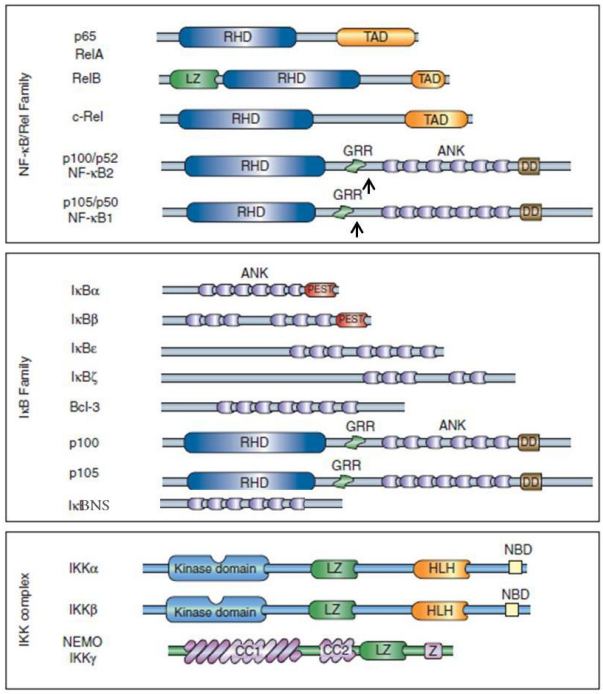

Figure 7. Schematic representation of NF-κB, IκB and IKK protein family. The mammalian Rel (NF-κB) proteins consist of five members: p65 (RelA), RelB, c-Rel and matures p52 and p50 protein, result of proteolytic processing (site of proteolysis are indicated by black arrows) from precursor proteins p100 and p105, respectively. All five proteins share a

conserved DNA-binding/dimerization domain called Rel Homology domain (RHD).The C-terminal halves of the p65, RelB and c-Rel proteins have transcriptional activation domains (TAD). In contrast, the C-terminal halves of the p52 and p50 proteins possess an ankyrin repeat-containing (ANK) inhibitory domain that is removed by proteasome-mediated proteolysis. A glycine-rich region (GRR), remaining after this process, is present in the mature forms. The IkB family consist of eight members, which are typified by the presence of multiple ANK and mediate binding to the RHD of NF-κB proteins, sequestering the complex in the cytoplasm. The IKK complex, composed by catalytic (IKKα or Ikβ) and regulatory (IKKγ/NEMO) subunits, phosphorylate the IkB proteins, signaling for ubiquitination and degradation.
The domains that characterize each protein are shown. CC, coiled-coil; DD, death domain; HLH, Helix loop helix; LZ, leucine-zipper; NBD, NEMO binding domain; PEST, proline-, glutamic acid-, serine-, and threonine-rich region; ZF, zinc finger. (From [86])

All members of the Rel/NF-κB family can associate to form distinct homo- or heterodimeric complexes. These different combinations form up to fifteen different dimers, but it has not yet been demonstrated the physiological existence and relevance for all possible dimeric complexes [86]. However, the p50-p65 heterodimer clearly is the most abundant in most cells, being found in almost all cell types [90].

Collectively, NF-κB transcription factor dimers bind to 9–10 base pair DNA sites, known as κB sites, in the promoters and enhancer regions of genes, thereby modulating gene expression [81]. Given that different NF-κB dimers have distinct DNA-binding site specificities for a collection of related κB sites, the combinatorial diversity of the NF-κB dimers contributes widely to the distinct transcriptional regulation [81].

As NF-κB has the ability to influence expression of numerous genes, also its activity is tightly regulated at multiple levels. The primary mechanism for regulating NF-κB is through proteins termed inhibitors of NF-κB (IκBs) and the kinase that phosphorylates it, the IκB kinase (IKK) complex [86, 90].

The IκB family members possess different affinities to individual NF-κB dimers and currently this family consists of eight members: IκBα, IκBβ, IκBε, IκBζ, BCL-3 (B-cell lymphoma 3), IκBNS, and the precursor proteins p100 and p105. They are characterized by the presence of five to seven ankyrin repeat motifs, which mediate their binding via protein–protein interactions with the RHD of NF-κB proteins in the cytoplasm, thereby making them transcriptionally inactive (figure 7). By the presence of this motifs, it is understood why the precursors p105 and p100 have their own internal inhibition [86, 88, 90], therefore, away from the nucleus, preventing DNA binding and gene regulation, or in other words, silencing their transcriptional activity [88].

A number of post-translational modifications also modulate the activity of the IκB and IKK proteins as well as NF-κB molecules themselves [86]. Nevertheless, other possible ways to regulate this pathways are already described such as the regulation of NF-κB by upstream IKK pathways [91].

3.2. Pathways of NF- κB: canonical and non-canonical

In most non-stimulated cells, NF-κB complexes are sequestered in the cytoplasm in an inactive form via IκB-bound complex. In recent years, it has become clear that there are at least two different pathways for NF-κB activation. The two major and best-described pathways are known as the canonical (or classical) pathway and the non-canonical (or alternative) pathway (figure 8). In both pathways, the common upstream regulator is the activation of an IκB kinase (IKK) complex, which consists of two catalytic kinase subunits (IKKα/IKK1 and/or IKKβ/IKK2) and/or a regulatory subunit, a scaffold called NF-κB essential modulator (IKKγ/NEMO). The differential requirement for IKK subunits, stimuli that leads to its activation and the activation of different NF-κB dimers define each one of these pathways.

The canonical pathway is representative of the general scheme of how NF-κB is regulated and activated. This is triggered by microbial products, such as lipopolysaccharides (LPS), flagellin or peptidoglycans [85], or proinflammatory cytokines, such as TNFα and IL-1, leading to activation of p65, c-Rel, RelB and p50 containing complexes [84]. In general terms, stimulation through receptors like cytokine receptors, such as Tumor Necrosis Factor-Receptor (TNFR) or IL-1 receptor (IL-1R), pattern recognition receptors (PRRs), such as Toll-like receptor 4 (TLR4) and antigen receptors, among many other stimuli, trigger signaling cascades that culminate in activation of IKK complex [88]. Firstly, upon recognition and binding of ligand to its cell surface receptor occurs the recruitment of adaptors (e.g., TRAFs, TNF receptor associated factors; and RIPs, Receptor interacting proteins) to the cytoplasmic domain of the receptor [81]. In turn, these adaptors often recruit an IKK complex that, in the canonical pathway, is composed of the α and β catalytic kinase subunits (IKKα and IKKß) and two molecules of the regulatory scaffold NEMO. Subsequently, the clustering of molecules at the receptor activates the IKK that phosphorylates the IκBs. This triggers IκB polyubiquitination, leading to its proteasome-dependent degradation and exposure of the NF-κB nuclear localization domain, which allows translocation of free NF-κB dimers to the nucleus and concomitant activation of specific target gene expression (figure 8). It is known that depending on the accessibility of the genome, regulated by epigenetic mechanisms, and the cell type, thousands of different target genes can be transcriptionally activated [82].

Alternatively, the non-canonical pathway (figure 8) is largely for activation of RelB/p100 complexes resulting of RelB/p52 complexes, occurring during the development of lymphoid organs responsible for the generation of B and T lymphocytes, dendritic cell activation and bone metabolism [81]. A small and certain number of receptor signals are known to activate this pathway, such as Lymphotoxin β (LT β), B-cell activating factor (BAFF), CD40 ligand (CD40L) and receptor activator for NF-κB ligand (RANKL), but not

TNFα [84, 89]. In contrast, the IKK complex involved contains two IKK subunits, but not NEMO. In this pathway, the ligand induced activation of NF-κB-inducing kinase (NIK) phosphorylates and activates an IKKα complex that, in turn, leads to phosphorylation of p100 at its C-terminal, where are the ankyrin repeats [81], resulting in its partial proteolysis to p52, followed by ubiquitination, degradation and finally liberation of the p52/RelB complex [86].

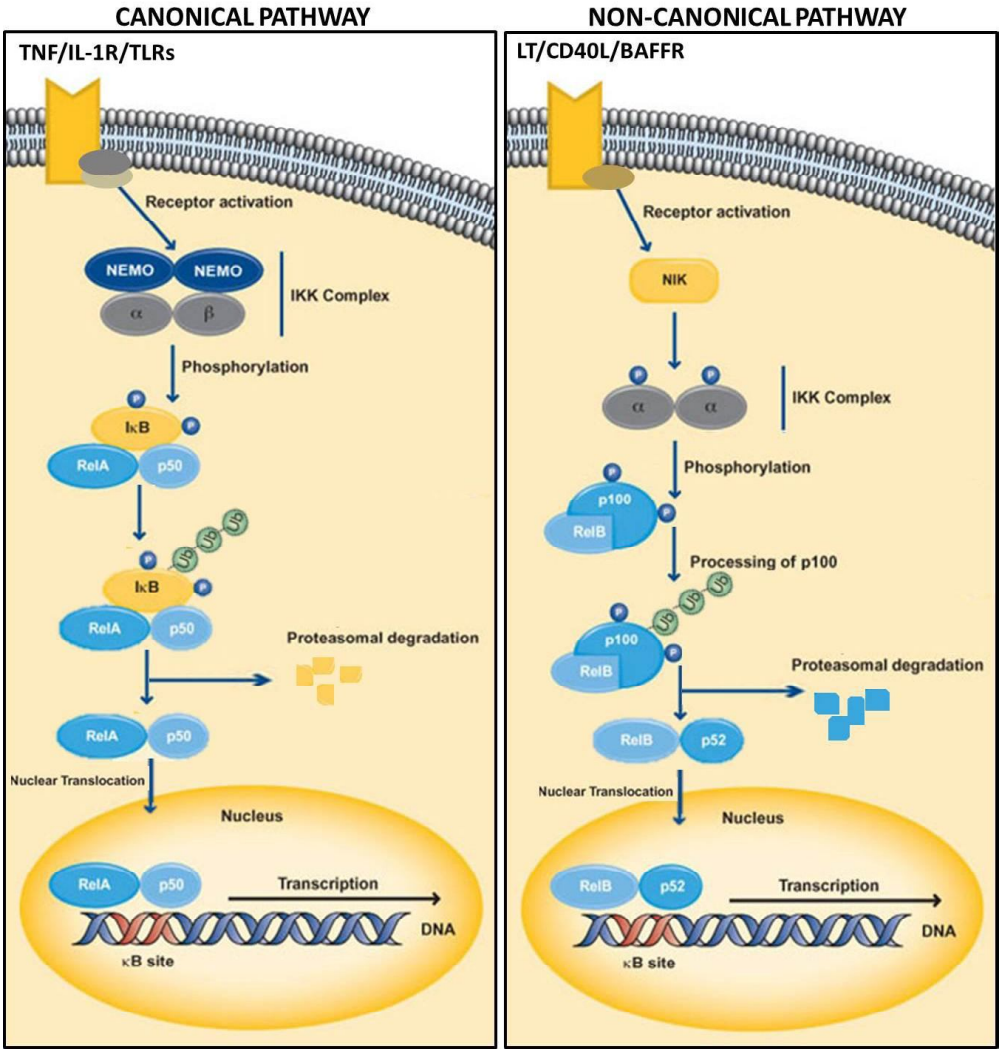

Figure 8. NF-κB signal transduction pathways. The canonical (or classical) NF-κB pathway is typically triggered by microbial products and proinflammatory cytokines, leading to activation of p65, c-Rel, RelB and p50 containing complexes. The binding of a ligand to a cell surface receptor (e.g., tumor necrosis factor-receptor, TNFR; Interleukin-1 receptor; or Toll-like receptors, TLRs) leads to recruitment of adaptors molecules (e.g., TRAFSs and RIP) to the cytoplasmic domain of the receptor. In turn, occurs the recruitment and activation of an IKK complex comprising IKK alpha and/or IKK beta catalytic subunits and two molecules of NEMO, in contrast with non-canonical composed only by two subunits IKK alpha. IKK then phosphorylate IκB leading to ubiquitination and degradation by the proteasome. The released NF-κB dimers translocate to the nucleus to turn on target genes. In contrast, the non-canonical (or alternative) pathway is largely for activation of p100/RelB complexes. It is activated by specific receptor signals, such as Lymphotoxin β (LT), B-cell activating factor (BAFF) or CD40 ligand, which leads to the activation of NF-κB inducing kinase (NIK), that phosphorylate and activates an IKK alpha complex. In turn, the p100 protein is phosphorylated and is lead to proteosomal processing, generating p52/RelB dimers. The activation of this dimers allows their translocation to the nucleus and targeting of specific κB elements. (Adapted from [92])

Despite of the large development in the comprehension of the complex network of NF-κB signaling pathway, some mechanisms are still to be understood. The continuing interest into the regulatory mechanisms that govern the activity of this transcription factor can be easily explained by its association with several number of diseases in which dysregulation of NF-κB has been implicated [86].

3.3. Targeting NF-κB in diseases

As mentioned early, in addition to the pivotal role in the control of genes involved in the immune and inflammatory response, the IKK/NF-κB pathway has gained recognition for its role as a key mediator in several diseases, namely inflammatory and metabolic disorders as well as in the development and progression of human cancers [84]. Although NF-κB activity is inducible in most cells, it can also be detected as a constitutively active nuclear protein, in certain cell types [82]. Some examples are mature B cells, neurons, and vascular smooth muscle cells [82], as well as a large number of tumor cells [86], such as AML [93], therefore, it can exert a variety of pro-tumorigenic function [82]. For example, it regulates genes involved in the inhibition of apoptosis, including the tumor necrosis factor receptor-associated factors (TRAF1 and TRAF2), the cellular inhibitors of apoptosis (c-IAP1/2 and XIAP) or Bcl-XL [94]. Some of these antiapoptotic stimulus block the activation of caspases involved in the apoptotic pathway [95], contributing to cell survival and consequently progression of cancer. Regardless the diverging opinions in the last years, around the double and opposed role of NF-κB in cellular apoptosis [96], several studies prompted investigators to the targeting of NF-κB pathways compounds, namely inhibition of its activity, as therapeutic approach to control cell disorders that contribute to these diseases.

The progress made in the understanding of the activation of the NF-κB pathway, as well as in the function performed by its core elements, such as NF-κB dimers, IκB proteins and the IKK complex, allowed the development of NF-κB pathway inhibitors aimed to control its dysregulation in several diseases. Some of these compounds include protein kinase inhibitors, inhibitors of NF-κB-mediated reporter gene expression, also called transactivation inhibitors, and proteasome inhibitors [96]. Despite the success achieved by many of these inhibitors, some limitations, such as the non-exclusive action in this pathway, have contributed to the need for new agents that can modulate and be more effective at targeting NF-κB for therapeutic use [96] .

CHAPTER II

PROJECT AIMS

AIP56 is classified as an AB-type toxin. This protein possess a N-terminal A domain, which displays metalloprotease activity by cleaving NF-κB p65 and, consequently, triggering apoptosis of phagocytes in sea bass, connected by a disulphide bridge to a C-terminal B domain, involved in the binding and internalization of the toxin into the host cells. So far, the boundaries of both the translocation and the receptor binding domain with the host cells remain undefined. However, bioinformatics analysis of AIP56's secondary structure and preliminary results from our group suggest the existence of a C-terminal subdomain that may correspond to a receptor binding region. Finally, studies in our lab showed that AIP56 is able to cleave NF-κB p65 and trigger apoptosis of mouse bone marrow derived macrophages in a similar way as previously described for fish macrophages. Therefore, in addition of what is known about not only the involvement of NF-κB in human pathologies but also the structural arrangement of AIP56, the clinical interest of using AIP56 as a biomedical tool is greatly potentiated.

With the above in mind, this work aimed at directing AIP56 chimeric toxins to cells other than phagocytes through the replacement of AIP56 putative receptor-binding domain with protein ligands, whose receptors are over-expressed in cancer cells, leading to the promotion of specific targeting, internalization, and catalytic activity of AIP56 towards specific cancer cells.

To achieve this goal, the following strategies were followed:

i) Construction of two chimeric proteins, containing AIP56 toxin in which the putative receptor binding domain (G374 to N497) was replaced by either α-melanocyte stimulating hormone (AIP56αMSH) or human InterLeukin-3 (AIP56-IL3)

ii) Purification of the protein chimeras

iii) Analysis of the toxicity induced by these proteins in specific cancer cells (acute myeloid leukemia (AML) cells and melanoma cells)

CHAPTER III

MATERIAL AND METHODS

1. Recombinant proteins

1.1. AIP56-IL3 and AIP56αMSH

1.1.1. Plasmid Construction

In this work, two chimeras were constructed: (i) AIP56-IL3, where the AIP56 predicted binding domain (amino acid G374 to N497) was replaced by human interleukin 3 (IL-3) and (ii) AIP56αMSH, where the AIP56 predicted binding domain was replaced by α-melanocyte stimulating hormone (αMSH). A scheme representing the cloning strategy is shown in figure 9.

AIP56-IL3: This construction was obtained by molecular cloning techniques using restriction enzymes for cleaving and inserting the coding sequence into the expression plasmid (figure 9A). The sequence encoding AIP56$^{1-373}$ was amplified from a plasmid containing the full length sequence of AIP56 (pETAIP56H^{+}) already available [74] using primers AIP56FW9NdeI and AIP56RV7SacI (table 3). Primer AIP56FW9NdeI introduces to the N-terminal of AIP56$^{1-373}$ an NdeI restriction site, for cloning into pET28a(+) vector (Novagen) in frame with a 6x His-tag, followed by a TEV (*Tobacco etch virus*) cleavage site, for affinity purification and tag removal upon protein expression. Primer AIP56RV7SacI introduces a SacI restriction site at the C-terminal of AIP56$^{1-373}$, for ligation to IL-3. The sequence encoding IL-3$^{1-132}$ [97] was amplified from a plasmid containing mature human IL-3 cDNA (pLX304-IL3; Dana-Farber/Harvard Cancer Center DNA Resource Core, Harvard Medical School) using primers IL3FW1SacI and IL3RV1XhoI (table 3). Primer IL3FW1SacI introduces to the N-terminal of IL-3$^{1-132}$ a SacI restriction site for ligation to AIP56$^{1-373}$. Primer IL3RV1XhoI introduces to the C-terminal of IL-3 a XhoI restriction site for cloning into pET28a(+). After an initial denaturation step for 2 minutes (min) at 94°C, AIP56$^{1-373}$ and IL-3$^{1-132}$ were amplified by 30 cycles of denaturation at 94°C for 45 seconds (sec), annealing at 66°C for 30 sec and extension at 72°C for 30 sec or 20 sec, respectively, followed by a final extension at 72°C for 5 min. The PCR-generated DNA fragments were extracted and purified from agarose gel using the GFX PCR DNA and Gel band purification Kit (GE Healthcare), following the company's instructions, and cloned into the pGEM-T Easy vector (Promega) using T4 DNA Ligase (Promega), at 4ºC overnight. After amplification, the 1.1 kb AIP56 and 0.4 kb IL3 DNA fragments were excised from pGEM-T Easy vector with NdeI and SacI or SacI and XhoI, respectively. AIP56$^{1-373}$ was first ligated into pET28a(+), previously digested with NdeI and SacI, yielding the intermediate plasmid pETAIP56$^{1-373}$, to which IL-3 was ligated after digestion of pETAIP56$^{1-373}$ with SacI and XhoI, yielding the final plasmid pETAIP56-IL3.

AIP56αMSH: This construct was obtained by two consecutive PCR reactions (figure 9B). The sequence encoding AIP56$^{1-373}$ was amplified from pETAIP56H+ [74] with primers AIP56FW9NdeI and AIP56αMSHRV1 (table 3), the last one adding part of αMSH to the C-terminal end of AIP56$^{1-373}$, yielding the template to the second reaction. In the second reaction, primers AIP56FW9NdeI and AIP56αMSHRV2 (table 3) were used to obtain AIP56αMSH final sequence. Primer AIP56FW9NdeI introduces an NdeI restriction site in frame with a 6x His-tag, followed by a TEV cleavage site, as explained above. Primer AIP56αMSHRV2 introduces the remaining sequence of αMSH followed by a XhoI restriction site, for cloning into pET28a(+). Both reactions were performed in the conditions as described above for AIP56-IL3, with exception of the annealing cycles: two initial cycles at 54°C and 30 subsequent cycles at 68°C. The 1.2 kb AIP56αMSH fragment was then extracted, purified, subcloned into the pGEM-T Easy vector and cloned into pET28a(+), as described for AIP56-IL3, but using NdeI and XhoI restriction enzymes, yielding plasmid pETAIP56αMSH.

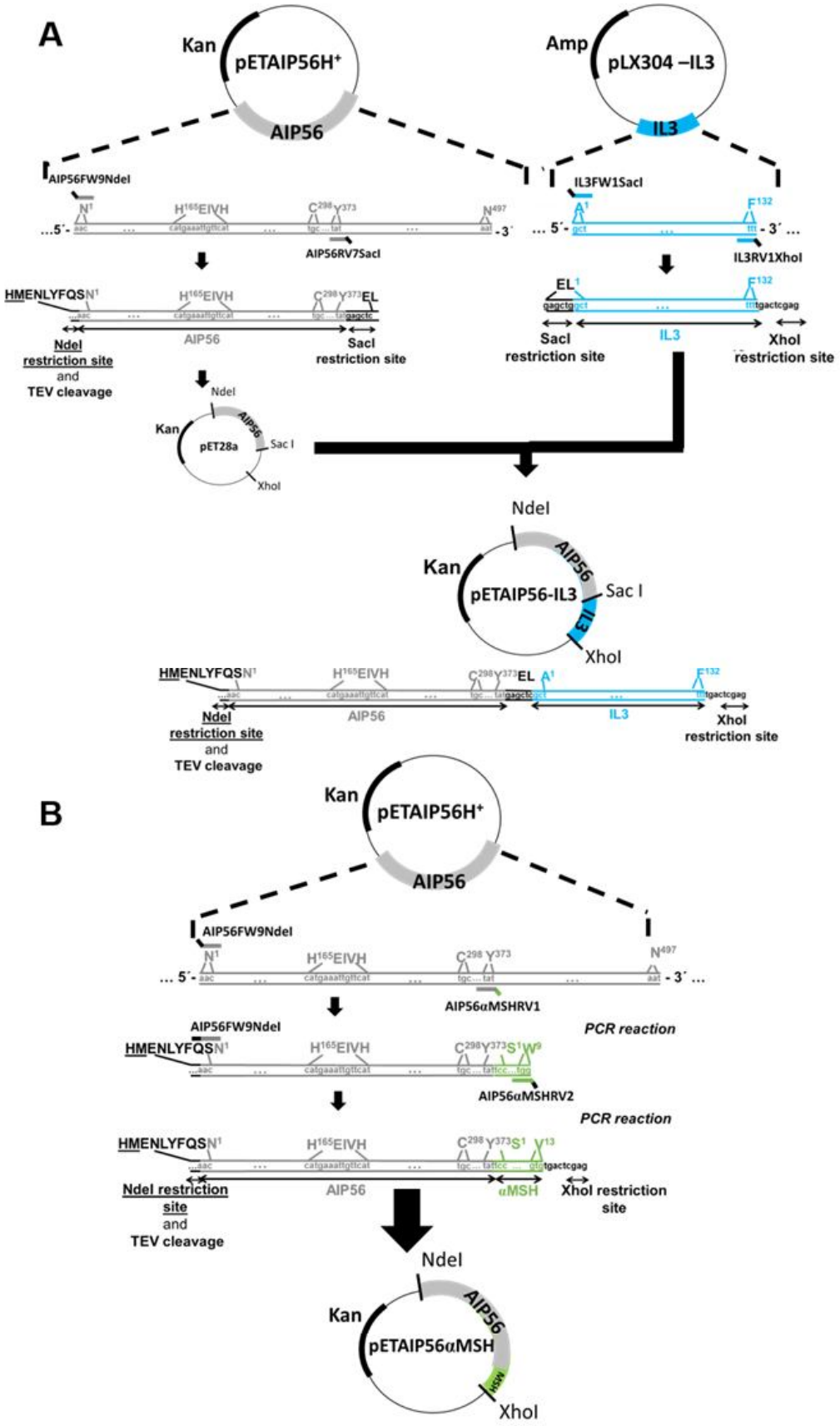

Figure 9. Schematic representation of the cloning strategy. A) Strategy used to obtain the construct pETAIP56-IL3. This construction was obtained by molecular cloning techniques and restriction enzymes. PCR reaction was performed with the

respective primers for amplification of AIP56 and IL3 sequences. After, it was cloned into pGEM®-T Easy vector in the presence of T4 DNA Ligase. The AIP56 DNA fragments and IL3 DNA fragments were digested with respective restriction enzymes and ligated into pET28a previously cut with NdeI and XhoI. Induction of E.*Coli* with this plasmid resulted on production of chimeric AIP56-IL3 protein. B) Strategy used to obtain the construct pET28AIP56αMSH. Chimera AIP56αMSH was obtained by two consecutive PCR reactions. The first reaction yielded the template to the second reaction. PCR reaction was performed with the respective primers for obtain AIP56αMSH final sequence. After, it was cloned into pGEM®-T Easy vector in the presence of T4 DNA Ligase. The AIP56αMSH DNA fragments were digested with NdeI and XhoI and cloning into pET28a previously cut with NdeI and XhoI. The primers used are indicated in respective figure.

All PCR reactions were executed in a Thermo Scientific Piko Thermal Cycler (Thermo Scientific), following the manufacturer´s instructions. PCR amplification was performed with 20 ng of DNA template, 25 mM $MgCl_2$, 10 mM deoxynucleotide triphosphates (dNTP) mix, 0.50 µg/mL of each primer, 1x PCR buffer (Promega) and 1 unit of Taq DNAPolymerase (Promega).

The plasmids obtained were produced in quantity and isolated from *E. coli* XL1 Blue, grown in Lysogenic Broth (LB) [98] supplemented with 50 µg/mL kanamycin (Kan), using QIAprep Spin Miniprep kit (Qiagen) following the manufacturer´s instructions. Transformants with the desired construct were identified by PCR, using primers T7 (forward) and T7 terminator (reverse) of pET28a(+), and sequencing. DNA concentration was determined by measuring the absorbance at 260nm (A260) using nanodrop.

Table 3. Primers used in this work.

Primer designation	Nucleotide sequence 5´- 3´	Construct
AIP56FW9NdeI	ccccatatggagaatctttattttcagagcaacaacgataaaccagatgcaagc	AIP56αMSH / AIP56-IL3
AIP56RV7SacI	gggagctcatatagaccggaattgagc	AIP56-IL3
IL3FW1SacI	ccggagctcgctcccatgacccagacaacg	AIP56-IL3
IL3RV1XhoI	gcgctcgagtcaaaagatcgcgaggctc	AIP56-IL3
AIP56αMSHRV1	cagcgaaaatgttccatggaataggaatatagaccggaattgagccc	AIP56αMSH
AIP56αMSHRV2	gcgctcgagtcacaccggtttgccccagcgaaaatgttccatgg	AIP56αMSH

1.1.2. Expression of chimeric AIP56-IL3 protein

To produce AIP56-IL3 protein, different strategies were attempted (figure 10).

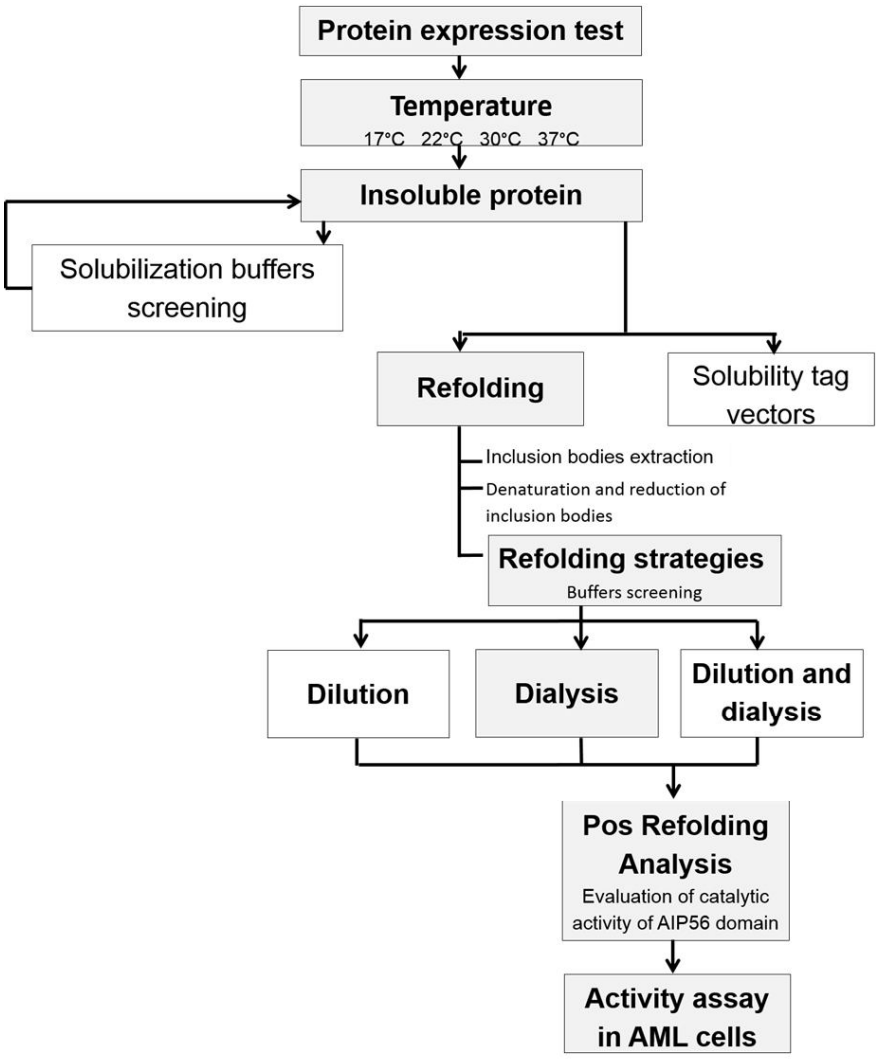

Figure 10. Flowchart of the strategy used in this work to obtain AIP56-IL3 recombinant protein. Protein production was attempted at different temperatures. However, it was always obtained in insoluble state. In order to solve this problem, different approaches were used: screening of different lysis buffers, using of vectors with solubility tags and refolding attempts. Highlighted in gray is the path from which it was possible to obtain protein to activity assays.

For expression of AIP56-IL3 protein, *E. coli* BL21(DE3) competent cells were transformed with 3 ng of plasmid pETAIP56-IL3 by the heat and shock method. Briefly, DNA and competent cells were incubated on ice for 30 min. After, the cells were heat-shocked for 90 seconds at 42°C, and immediately incubated on ice for 2 min. Afterwards, 4 volumes of Super Optimal broth medium with catabolite repression (SOC) medium (2% Tryptone, 1% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM Glucose) was added and incubated at 37ºC for 45 min, with shaking at 150 rotations per minute (rpm). Finally, transformants were plated in LB-agar plates containing 50 µg/ml Kan and incubated overnight (ON) at 37ºC. A single colony was inoculated into LB medium supplemented with 50 µg/ml Kan and incubated ON at 37°C with shaking (150 rpm). The culture was then refreshed in fresh medium containing Kan, using 1:100 dilution

of the culture grown ON. When the culture reached an OD of 0.4 at 600 nm was incubated at different temperatures (17°C, 22°C, 30°C or 37°C), with shaking (150 rpm). Recombinant protein expression was induced by adding 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), when the culture reached an OD of 0.6 at 600 nm, and protein expression evaluated at different temperatures (17°C, 22°C, 30°C or 37°C) and time-points (see respective figure in results section). Cells were then harvested by centrifugation at 4000 g for 30 min at 4°C (Beckman Avanti J-26 XP in an AJ81000 rotor), resuspended in lysis buffer (50 mM NaP, 500 mM NaCl, 200 µg/mL lysozyme, 1:500 PMSF) on a ratio of 35 mL of lysis buffer per 1 L of culture and stored at -20°C. Induced bacterial cells were lysed by freeze/thaw, followed by sonication on ice (*duty cicle*- constant; *output control*- 5; 15x10 seconds) in the presence of 10 mM $MgCl_2$ and 10 µg/mL DNAse I. The soluble and insoluble fractions of the total lysates were separated by centrifugation at 21000 g for 15 min at 4°C and analyzed by SDS-PAGE followed by Coomassie-blue R-250 (Sigma Chemical) staining.

As soluble AIP56-IL3 protein has not been obtained with any of the induction temperatures tested, a strategy based on adding different additives, described as promoting recombinant protein stability [99], to a base lysis buffer was attempted. The resulting lysis buffers are listed in table 4.

After induction at 30°C, as described above, pellet from 1 mL of *E. coli* cell culture was resuspended in 450 µL of fresh lysis buffer and 50 µL of one of the eleven additives (table 4), previously prepared in 25 mM HEPES pH=7.0, at a final additive concentration indicated in table 4. Afterwards, sample was incubated on ice for 30 min, sonicated on ice (*duty cicle*- constant; *output control*- 4; 5x7 seconds) and the total cell lysate centrifuged at 4000 g for 30 min at 4°C. Then, supernatant and pellet were taken and stored as soluble and insoluble fractions, respectively. Both fractions were analyzed by Coomassie-blue stained SDS-PAGE.

Table 4. List of solubilization additives and buffer at final concentrations used in the assay to try rescuing soluble protein.

Fresh lysis Buffer	Solubilization Additives	
25 mM HEPES pH=7.0, 500 mM NaCl, 10 % w/v glycerol, 0.5 % CHAPS, 10 mM $MgCl_2$, 0.1 % Lysozime, 25 U/mL Benzonase	0.75 M Trehalose	0.375 M L-Arginine
	1 M Xylitol	0.1 M Potassium citrate
	0.5 M Mannitol	0.1 M Dipotassium phosphate
	1 M Glycine betaine	0.01 M Sodium selenite
	1 M Trimethylamine N-Oxide	1 M 3-(1-Pyridinio)1-propane sulfonate

1.1.3. Purification of AIP56-IL3

For obtaining soluble recombinant AIP56-IL3 from the insoluble fraction, a refolding protocol has been optimized from a protocol previously developed for obtaining a soluble truncated form of AIP56 [74]. The correct folding of AIP56-IL3 protein was then evaluated by (i) a catalytic assay that, by detecting catalytic activity, indicate the correct folding of the AIP56 domain, and (ii) an internalization assay that, by detecting receptor binding capacity, indicate the correct folding of the IL3 domain.

1.1.3.1. Preparation of inclusion bodies

After induction in BL21 *E. coli* at 30°C for 2 h, as described before, AIP56-IL3 protein was produced as inclusion bodies. Thus, AIP56-IL3 inclusion bodies were isolated from *E. coli* cell pellets by sonicating twice in cold isolation buffer I (20 mM NaP pH 7.4, 500 mM NaCl, 0.5 mM EDTA, 5 % Glycerol, 2 % Triton, 2 M Urea) followed by centrifugation at 22000 g for 15 min at 4°C, and once in cold isolation buffer II (20 mM NaP pH 7.4, 500 mM NaCl, 5 % Glycerol). After centrifugation, as above, pellets were stored at -20°C.

1.1.3.2. Denaturation and reduction of inclusion bodies

After thawing, inclusion bodies were solubilized in 8 M urea, 20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 1 mM 2-mercaptoethanol, in a ratio of 4 mL of buffer per 1 g of the original cell pellet, for 1 h at room temperature with gently stirring, followed by centrifugation at 21000 g for 15 min at 4°C. The supernatant fraction was withdrew and the denatured AIP56-IL3 protein adjusted to 0.2 mg/mL and subjected to refolding. Solubilized inclusion bodies were evaluated by Coomassie-blue stained SDS-PAGE.

1.1.3.3. Refolding

Three different strategies of refolding were attempted, as shown in figure 10 and described below.

i) Refolding by dialysis

Denatured AIP56-IL3 was dialyzed (12-14000 molecular weight cut-off; dialysis Membrane Spectrumlabs) three times (6h + overnight + 6h) under stirring at 4°C against 50 volumes of one of the buffers described in table 5.

ii) Refolding by dilution

Denatured AIP56-IL3 was added to one of the refolding buffers described in table 5 by drop wise under rapid stirring at 4°C.

iii) Refolding by dilution and dialysis

Alternatively, denatured AIP56-IL3 was added to one of the refolding buffers described in table 5 by drop wise under rapid stirring at 4°C until urea concentration reached 1 M. Urea was then decreased to insignificant concentrations by performing an overnight dialysis at 4°C as described above.

Table 5. Buffers used in the refolding protocol.

Buffer refolding	Buffer preparation
PBS with 10 %Glycerol	10 mM $NaH_2PO_4 \cdot H_2O$ pH=7.2, 150 mM NaCl, 10 % Glycerol
IL-3 [100]	0.5 M L-Arginine HCl, 100 mM Tris pH=8, 0.9 mM oxidized glutathione
Tris-NaCl	20 mM Tris pH=8, 200 mM NaCl
Tris-NaCl with 10% Glycerol	20 mM Tris pH=8, 200 mM NaCl, 10 % Glycerol

1.1.4. Analysis and storage

After refolding and clarification by centrifugation (21000 g for 5 min) the supernatant containing AIP56-IL3 were rapid freeze in liquid nitrogen and kept at -80ºC until use. Refolded AIP56-IL3 was analyzed by Coomassie-blue stained SDS-PAGE. Protein concentration was quantified by measuring absorbance at 280nm using nanodrop.

1.2. Sea bass p65 (sbp65) Rel ^{35}S-labeled protein

For testing the AIP56-IL3 cleavage activity, the sea bass NF-κB p65 Rel homology domain was synthetized *in vitro* using a rabbit reticulocyte lysate transcription/translation system (TNT T7 Quick Coupled Transcription/Translation systems kit, Promega, TM045) following the manufacturer´s instructions. Briefly, the reaction containing 1 µg of plasmid pET28a(+) encoding sbp65 Rel, TNT T7 Quick Master mix (containing Rabbit Reticulocyte Lysate) in the presence of Redivue™ L-[^{35}S]-Methionine (specific activity of N1000 Ci/mmol) was incubated for 90 min at 30°C and stored at -80°C. The result of translation and radiolabel incorporation were evaluated by 14 % SDS-PAGE, transferred to nitrocellulose membrane (as described below) and revealed by autoradiography after 48 h film exposure.

2. Cells

AML cells. In this work, three human cells lines of Acute Myeloid Leukemia (AML) have been used: HL-60, NB-4 and KG-1 (Table 6).

Table 6. Classification of cell lines of acute myeloid leukemia (AML) used in this work, according to French-American-British (FAB) classification system [101], and their relevant characteristics.

Cell line	FAB subtype	Description FAB	Origin	Characteristics
HL-60	M2	Acute myeloblastic leukemia, with maturation	1976, 35 year old female [102]	• Doubling time: 30h to 40h • Myeloblastic morphology • 2 to 4 rounded nuclei [102] [103]
NB-4	M3	Acute promyelocytic leukemia (APL)	1989, 23 year old female [104]	• Doubling time: 36h to 40h [104] • Round and polymorphic cells
KG-1	M6	Erythroleukemia	1977, 59 year old male [103]	• Doubling time: 40-50h • Morphology similar to dendritic myeloid cells [103]

Human leukemia cell lines HL-60, NB-4 and KG-1 were kindly provided by Drª Paula Ludovico, University of Minho. All cells were grown in culture suspensions. NB-4 and HL-60 cells were routinely cultured in RPMI 1640 medium supplemented with L-glutamine (Thermo Scientific, ref.SH30255.01) supplemented with 10 % heat inactivated fetal bovine serum (FBS) (Life Technologies, ref.10500064) and 1 % Penicillin/streptomycin (PenStrep; Lonza). KG-1 were cultured in the same culture conditions but supplemented with 20 % FBS. All cells were kept at 37°C in a 95 % air and 5 % CO_2 humidified incubator. For these experiments, cells were used between 6 and 20 passages.

3. Functional assays

As the AIP56-IL3 chimera results from the fusion of two different and independent functional domains, a catalytic and putative translocation domain, provided by AIP56, and a receptor-binding domain, provided by IL3, assays for testing the catalytic and receptor binding activities, as well as intoxication, have been developed.

3.1. Catalytic assays

Using in vitro translated p65. In order to verify the catalytic activity of the AIP56 domain, different concentrations (see respective figure in results section) of refolded AIP56-IL3 were incubated (2 h at 22°C) with in vitro Rabbit Reticulocyte Lysate translated ^{35}S-labeled sbp65Rel, in each respective refolding buffer (table 5), in a final volume adjusted to 20 µL. A similar reaction but without AIP56-IL3 was used as control. The cleavage of NF-κB p65 was assessed by resolving in 14 % SDS-PAGE, transferred to nitrocellulose membrane (as described below) and revealed by autoradiography after 48h film exposure.

Using native p65. The catalytic activity of refolded AIP56-IL3 was also assessed towards native p65 obtained from HL-60 cell lysates. For this purpose, cell lysates were first obtained by incubating cells with lysis buffer (10 mM Tris-HCl pH 8.0, 0.5 % (v/v) Triton X-100, 150 mM NaCl and 10 % (v/v) glycerol) for 30 min on ice (30 µL per $2.0x10^6$ cells), briefly sonicated and centrifuged at 21000 g for 10 min at 4ºC. The soluble fraction was quickly stored at -80°C.

For evaluating the AIP56-IL3 catalytic activity, aliquots of $2.0x10^5$ cell lysates were incubated with AIP56-IL3 or recombinant AIP56 (positive control) in the concentration indicated (see respective figure in results section) for 2 h at 22°C, in a final volume of 30 µL. Mock treated cell lysates were used as negative control. The cleavage of NF-κB p65 was assessed by Western blotting resolved in 10 % SDS-PAGE using a specific antibody to p65 (sc-372, Santa Cruz Biotechnology), as described below in section 4.

3.2. Intoxication assays

Using NF-κB p65 cleavage as read out. AML cell suspensions were adjusted to a density of $1.0x10^6$ cells, plated in wells of 24-well plates and incubated for different times (see respective figure in results section) at 37ºC (humid atmosphere with 5 % CO_2) with AIP56-IL3 or recombinant AIP56, in concentration indicated (see respective figure in results section), in a final volume of 250 µL or 1 mL when incubated for more than 24 h. When specified, cells were pre-treated for 1 h and incubated in the presence of 20 µM Proteasome inhibitor MG132 (Enzo Life Science).

After incubation, cells were centrifuged at 285 g for 5 min at 4ºC, washed once with PBS (10 mM NaH_2PO_4 H_2O pH=7.2, 150 mM NaCl) and lysed with 60 µL of GLB (1x) and 100 mM DTT. NF-κB p65 cleavage was analyzed by Western blotting using a specific antibody to p65 (sc-372, Santa Cruz Biotechnology).

Using apoptosis as read out. Apoptogenic activity was evaluated by the presence of typical morphological signs of apoptosis, namely, nuclear fragmentation, chromatin condensation and cell blebbing with formation of apoptotic bodies [105]. Apoptosis was assessed during different time points (2 h, 4 h, 6 h, 24 h, 48 h) after incubation at 37ºC with AIP56-IL3 or recombinant AIP56 at 70, 175 or 650 nM. AML cell suspensions were first fixed with 10 % of 37 % formaldehyde in absolute ethanol for 1 min, washed once with water and stained with Hemacolor (Merck) as described in [71, 74]. Morphology was evaluated by light microscopy.

Using the LF-PA system from *Bacillus anthracis* as delivery system. As an alternative approach for evaluating the effect of cleaving NF-κB p65 in AML cells, the

lethal factor-protective antigen (LF-PA) system from *Bacillus anthracis* was used for delivering the AIP56 N-terminal catalytic domain into the cells. For this purpose an LF·AIP56 chimeric protein produced in our laboratory [74] was used. LF·AIP56 consists on the N-terminal portion of anthrax lethal factor (LF^{11-263}) fused to AIP56 N-terminal catalytic domain (LF^{11-263}·$AIP56^{1-261}$). The anthrax protective antigen (PA), the binding receptor for LF, was kindly provided by Professor Dr. Cesare Montecucco (Dipartimento di Scienze Biomediche Sperimentali dell'Úniversità di Padova and Instituto di Neuroscienze del CNR, Padova, Italy).

Briefly, AML cells were plated at a density of $1.0x10^6$/well of a 24-well plate and incubated simultaneously with LF·AIP56 (20 nM) and PA (10 nM) for 2 or 4 h at 37ºC in a final volume adjusted to 500 µL. After, cells were centrifuged at 285 g for 5 min at 4ºC, washed once with PBS and lysed with GLB (1x) and 100 mM DTT. NF-κB p65 cleavage was evaluated by Western blotting, as described below in section 4, and apoptogenic activity analysed, after 4h incubation, as described above. This experiment was repeated but comparing to higher concentration of LF·AIP56 (40 nM) and PA (20 nM) and extending to 6 h incubation.

3.3. Internalization assays

For detection of AIP56-IL3 within cells an internalization assay has been performed. For this, cells were plated on 24-well plates at a density of $5x10^5$ cells/well, pulsed with 200 nM of AIP56-IL3 protein or recombinant AIP56 and incubated for 30 min on ice, for allowing the binding of the toxins to its cell surface receptor. After, cells were transferred to 37ºC and incubated for 10 min for allowing internalization of receptor-bound toxin into cells by activation of the endocytic machinery. The cells were then transferred again to ice for 5 min in order to block protein degradation, recycling and/or other cellular activities. After, cells were washed twice with ice-cold PBS, to remove unbound toxin, and incubated for 10 min on ice with 125 µg/mL Pronase E from *Streptomyces griseus* (Sigma Aldrich P5147) diluted in PBS (final volume of 250 µL), in order to eliminate extracellular cell-associated toxin ("shaving"). Next, Pronase E was inactivated with 1 mL of 250 µg/mL PMSF in PBS and washed twice with ice cold PBS. Cells not treated with Pronase E were used as control.

In order to check the efficiency of Pronase E in removing the extracellular cell-bound toxin a similar protocol was applied to cells where the 10 min internalization step at 37ºC has been replaced by 10 min incubation on ice for preventing internalization. Untreated, mock-treated cells and cells treated with protein but without incubation with pronase E were used as controls. Finally, cells were collected either by centrifugation or

by scrapping from the wells, lysed with 60 µL GLB (1x) and 100 mM DTT, and AIP56 detected by Western blotting using a rabbit anti-AIP56 antibody [70], as described below in section 4.

4. Miscellaneous

4.1. SDS-Page and Western Blotting

Proteins were mixed with Gel Loading Buffer (GLB; 50 mM Tris-HCl pH 8.8, 2% SDS, 10 % glycerol, 0.017 % bromophenol blue, 2 mM EDTA pH 8.8, 100 mM DTT) and denatured by heating at 95ºC for 5 min. Samples were run on a reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and either stained with Coomassie Blue R-250 (Sigma Chemical) or transferred to nitrocellulose membrane in transfer buffer (25 mM Tris-Base, 192 mM glycine, 20 % methanol, pH 8.3), for 1 h at 19 V. For controlling transfer efficiency, protein loading and locating the molecular weight markers, membranes were stained with Ponceau S (Sigma) and scanned. Membranes were then blocked with Tris-Buffered saline (TBS) with 0.01 % tween-20 (Sigma) (T-TBS) containing 5 % of non-fat dry milk at room temperature for 1 h or overnight at 4°C, to saturate (blocking) nonspecific binding sites and prevent non-specific binding of both primary and secondary antibodies. After, membranes were incubated for 1 h at room temperature (RT) with shaking with the corresponding primary antibody: anti-human NF-κB p65 C-terminal domain (c-20) rabbit polyclonal IgG antibody (sc-372, Santa Cruz Biotechnology; 1:3000), rabbit anti-AIP56 [70] (2^{nd} bleed, 1:5000) or anti-GAPDH (6C5) mouse monoclonal antibody (sc-32233, Santa Cruz Biotechnology; 1:20000), all diluted in blocking solution. Then, membranes were washed three times (5 min each) with T-TBS at RT and incubated with goat anti-rabbit alkaline phosphatase conjugated secondary antibody (A9919, Sigma Aldrich; 1:30000) or goat anti-mouse alkaline phosphatase conjugated secondary antibody (A2429, Sigma Aldrich; 1:10000) in T-TBS. The reaction was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Promega).

4.2. Biochemical parameters

Theoretical molecular weight was obtained using the ProtParam tool (http://web.expasy.org/protparam/).

4.3. Purity of the protein

The purity of the proteins was determined by densitometry analysis of Coomassie Blue-stained gels using Fiji software, and results expressed as density of the recombinant protein band relative to total density of the lane.

4.4. Protein quantification

Concentration of recombinant protein was determined by measuring absorbance at 280 nm using nanodrop.

CHAPTER IV

RESULTS

1. AIP56-IL3 was successfully cloned

To test the concept of using AIP56 fused to receptor-ligand moieties for targeting specific cells, two recombinant proteins were engineered, by replacement of the putative receptor binding domain of AIP56 (G374 to N497) either by α-melanocyte stimulating hormone (α-MSH) or Interleukin-3 (IL-3), and produced. The constructs consist on the first 373 amino acids of AIP56, including its catalytic domain, fused to α-MSH or to human IL-3, the later via a glutamic acid (E)-leucine (L) linker resulting from the SacI restriction site used to fuse AIP56 to IL-3.

For purification purposes, a 6xHis purification tag followed by a TEV cleavage site were added to the N-terminus, to avoid putative interferences in the cell binding ability assured by the C-terminal fused moiety, and for removing the purification tag. Constructs were inserted into pET28a(+), rendering pET28AIP56αMSH and pET28AIP56-IL3 expression vectors. Cloning was performed using molecular biology techniques. The correct sequences of the constructs were verified by sequencing analysis. An error (nucleotide deletion) has been introduced in the sequence of AIP56αMSH and, therefore, recombinant protein production and functional testing were only performed for AIP56-IL3 (see annex figure 1S). Constructs are schematically represented and compared with full length AIP56 in figure 11.

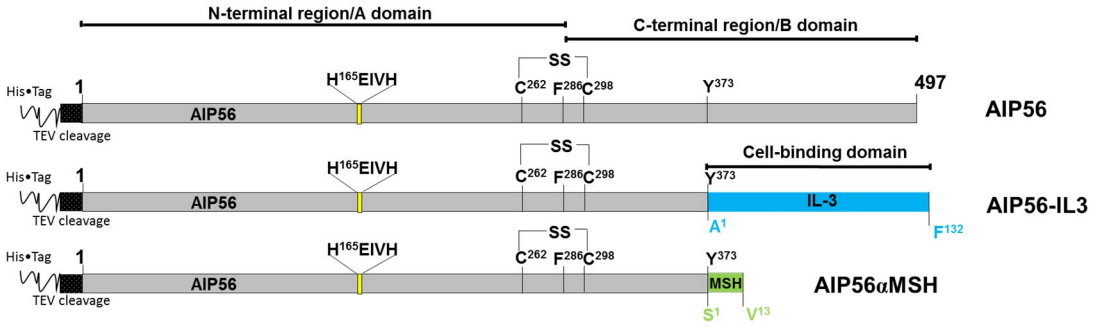

Figure 11. Schematic representation of chimeric proteins used in this work. Full length AIP56 (used as control in each experience), AIP56 fused with interleukin-3 (AIP56-IL3) and AIP56 fused with α-Melanocyte Stimulating Hormone (AIP56αMSH) are depicted.

2. AIP56-IL3 chimera is obtained in insoluble state

BL21 (DE3) *E. coli* were transformed with pET28AIP56-IL3. Production of recombinant protein was then attempted by screening and optimization of known protocols. Protein induction and solubility at different temperatures was assessed and monitored at different time points by SDS-PAGE.

Analysis of the SDS-PAGE gel showed that the recombinant protein was successfully obtained in three (22ºC, 30ºC and 37ºC) of four temperature tested (figure 12A); however,

they have been all obtained in insoluble state (figure 12B). Despite several attempts, induction did not occur at 17ºC. The induction temperature for posterior tests was 30ºC. The recombinant protein has a predictive molecular weight of 60.4 kDa.

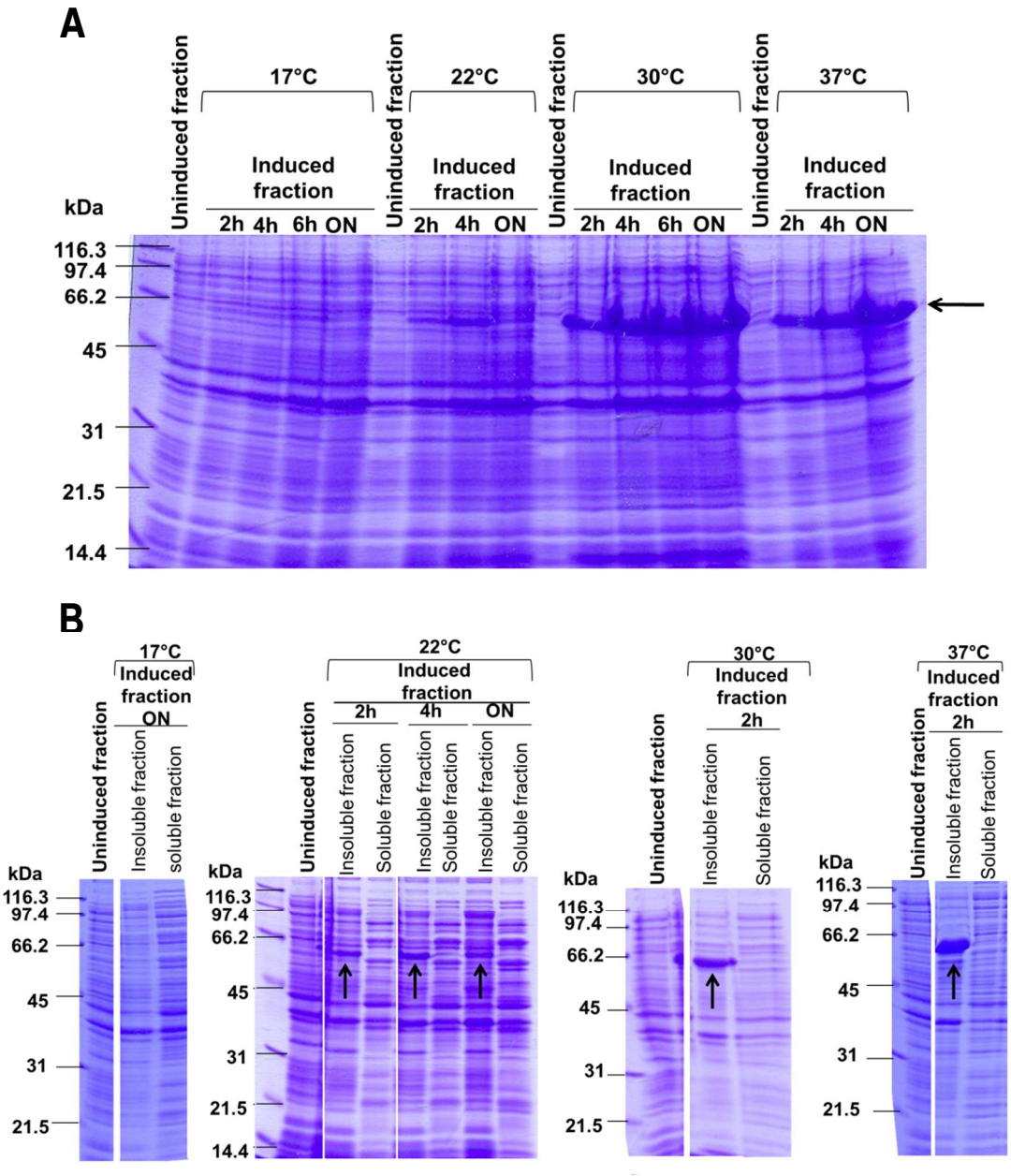

Figure 12. SDS-PAGE gels representing the induction temperature and solubility tests for AIP56-IL3. Culture of *E. coli* BL21(DE3) competent cells transformed with plasmid pETAIP56-IL3 were grown at indicated temperature. When culture reached an OD of 0.6 at 600 nm, recombinant protein expression was induced with 0.5 mM IPTG and incubated with shaking (150 rpm). A) The cells were harvested by centrifugation and the fraction of total lysates analyzed. Coomassie Blue staining of a 12 % acrylamide SDS-PAGE showing the induction of recombinant protein at different time points. The expected molecular weight of the recombinant protein (~60.4 kDa) is indicated by an arrow. Molecular weight standards with the corresponding weights are indicated. B) Coomassie Blue staining of a 12 % acrylamide SDS-PAGE showing the soluble (supernatant) and insoluble (pellet) fractions of induced protein. After induction and incubation for the indicated time, cells were harvested by centrifugation, resuspended in lysis buffer and stored at -20°C. Cells were additionally lysed by freezing/thawing, followed by sonication on ice. Soluble and insoluble fractions were separated by centrifugation and analyzed by Coomassie-blue stained SDS-PAGE. The expected protein band at ~60.4 kDa is indicated by an arrow.

In order to obtain soluble protein, a new screening was performed using different lysis buffers, in which eleven additives, described as recombinant protein stability promotors during cell lysis [99], have been selectively added. Cells were induced for 2 hours at 30 ºC and lysed with fresh lysis buffer (see material and methods section) with one of the mentioned additives (figure 13). Analysis by Coomassie-stained SDS-PAGE gel revealed that, for all conditions, recombinant proteins were in the insoluble fraction, precluding the use of the above mentioned additives for obtaining soluble AIP56-IL3 (figure 13).

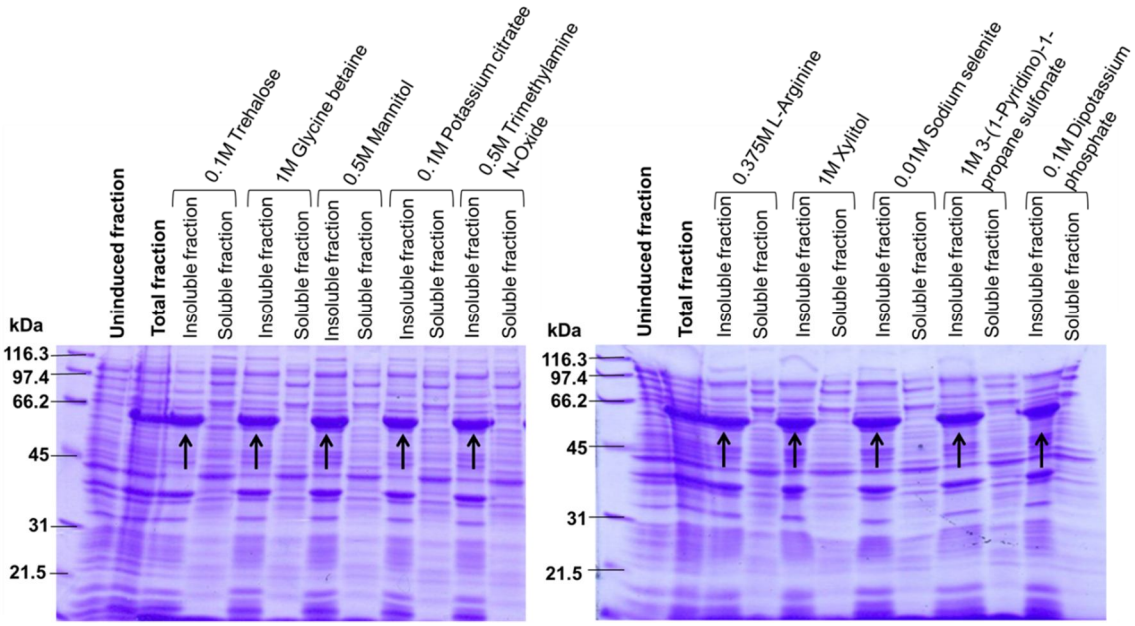

Figure 13. SDS-PAGE showing results from the lysis buffer screening. In each condition, cell culture pellets were lysed using a base lysis buffer(fresh lysis buffer) to which different additives have been selectively added (indicated on top of the gel). Subsequently, cells were sonicated and centrifuged, and the insoluble and soluble fractions analyzed by Coomassie Blue stained 12 % acrylamide SDS-PAGE. Arrows indicate the recombinant protein.

Therefore, two additional approaches were attempted: (i) cloning the chimeric proteins in vectors with solubility tags and (ii) refolding the insoluble protein previously obtained. The first strategy was performed by an undergraduate student and is not reported in this thesis.

3. Refolded AIP56-IL3 is catalytically active

Insoluble AIP56-IL3 was extracted from *E. coli* BL21 (DE3) cultures induced at 30°C for 2 hours. The general strategy used to recover active protein from inclusion bodies involved three main steps: (i) isolation and wash of inclusion bodies; (ii) solubilization of aggregated protein with 8 M urea (solubilizing agent); and (iii) refolding. The first two steps were performed based on an already established protocol. For the

refolding step, screening of different parameters, including refolding buffer and techniques, were attempted (table 7 and figure 14).

Table 7. Refolding parameters tested in this work.

Refolding buffers	Refolding techniques
PBS with 10% glycerol	Dilution
IL-3	Dialysis
Tris-NaCl	Dilution and dialysis
Tris-NaCl with 10% glycerol	

The choice of refolding buffers was based on prior experience with refolding of other AIP56-derived proteins [74]. In addition, the IL-3 buffer, reported in the literature and shown to successfully refold other chimeras containing fused IL-3 [100, 106], was included. However, the chelate constituent EDTA (Ethylenediaminetetraacetic acid) present in this buffer was removed, to avoid interference with the metalloprotease activity of AIP56.

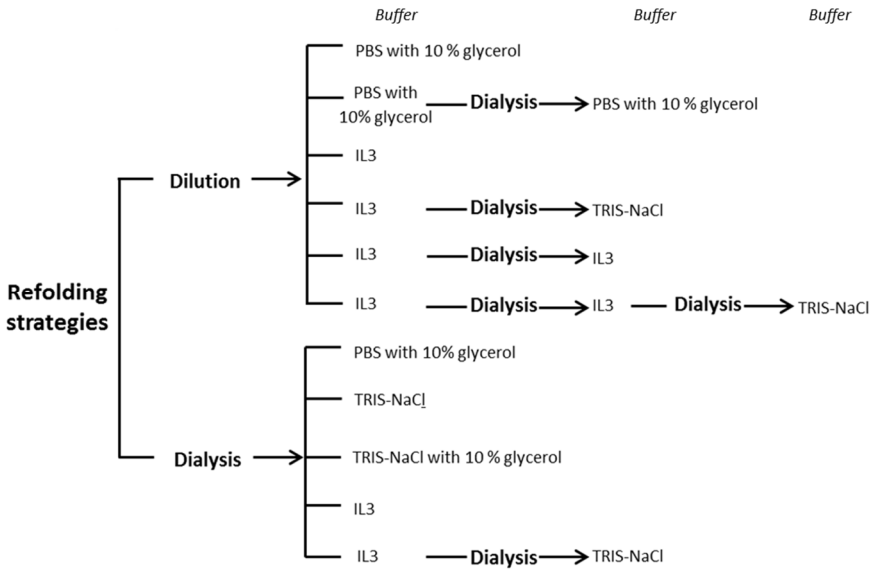

Figure 14. Combination of different refolding techniques with distinct refolding buffers tested. The initial step of the refolding process was done by dilution or dialysis to each respective buffer. For dilution, proteins were either diluted until removal of the denaturing agent or, alternatively, diluted until 1 M of denaturing agent has been reached followed by dialysis against the indicated buffer. For dialysis, proteins were either dialyzed against each respective buffer or, in the case of IL3 buffer, exchanged by dialysis to Tris-NaCl.

The purification of inclusion bodies resulted in a relatively high amount of insoluble AIP56-IL3 with low levels of other unspecific proteins. Since the solubilisation of this fraction yielded AIP56-IL3 with >80% purity (not shown), the refolding protocols were subsequently performed without further purification by nickel-affinity chromatography using the N-terminal 6xHis-tag.

Refolding efficiency, under the selected conditions, was done by analyzing equivalent volumes of soluble and insoluble fractions by SDS-PAGE (figure 15A and figure 3S in Annex). The best strategy was selected considering that recovery of protein in the soluble fraction is an indicative of a successful refolding. Direct dialysis against Tris-NaCl or IL-3 buffer as well as dilution followed by dialysis against PBS with 10 % glycerol (Figure 3S) seem to gather the best conditions for refolding this chimera. Due to its higher complexity (multi steps) without significant gain, the condition involving dilution and dialysis against PBS with 10 % glycerol was rejected.

The AIP56-IL3 protein encompasses the catalytic domain of the AIP56 toxin, which acts by cleavage of NF-κB p65 [107]. Therefore, the proteolytic activity of AIP56-IL3 towards p65 was evaluated, in order to confirm the correct folding of the AIP56 domain. For this purpose, refolded AIP56-IL3, after dialysis against Tris-NaCl or IL3 buffer, was incubated with ^{35}S-labeled sbp65Rel, and p65 cleavage assessed by autoradiography. AIP56 full length was used as control.

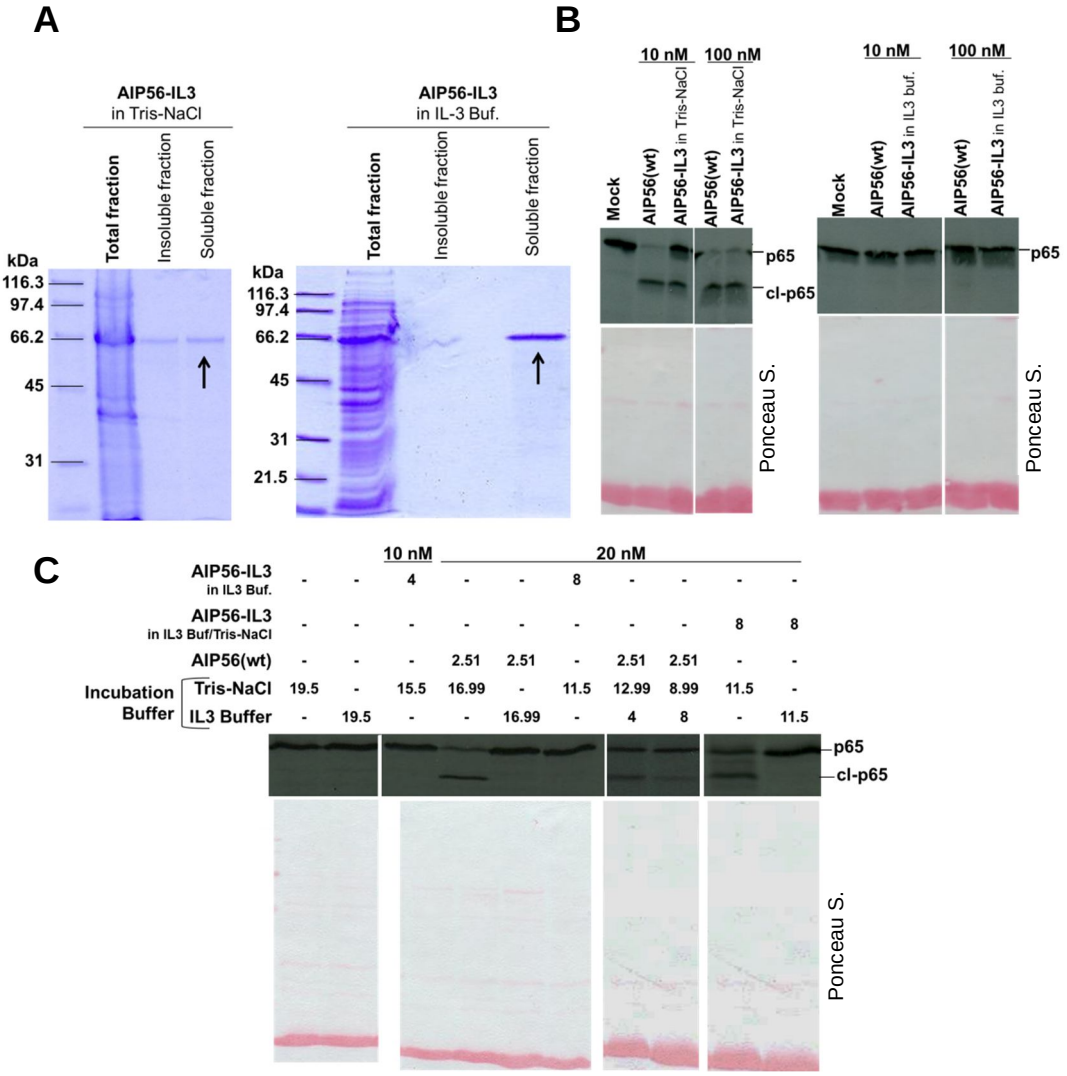

Figure 15. Analysis of the refolding process. A) Denatured AIP56-IL3 was dialyzed against Tris-NaCl (indicated in figure as: AIP56-IL3 in Tris-NaCl) or IL3 Buffer (indicated in figure as: AIP56-IL3 in IL3 buf.). The insoluble and soluble fraction of refolded AIP56-IL3 was analyzed by Coomassie-blue stained 12 % acrylamide SDS-PAGE. The overexpressed protein (~60.4 kDa) is indicated by an arrow. B) Catalytic activity of AIP56-IL3. Incubation of in vitro translated ^{35}S-labeled sbp65Rel with AIP56 in Tris-NaCl and AIP56-IL3 dialyzed against Tris-NaCl (AIP56-IL3 in Tris-NaCl) resulted in p65 cleavage. When AIP56-IL3 was dialyzed against IL3 Buffer (AIP56-IL3 in IL3 Buf) no cleavage was observed even at 100 mM. The mix was incubated for 2 hours at 22°C with 10 nM or 100 nM of the indicated protein and p65 cleavage assessed by autoradiography. The position of full length (p65) and cleaved (cl-p65) p65 are indicated. The corresponding Ponceau staining membranes are shown below. C) Inhibitory effect of IL3 buffer. The IL3 buffer from refolding of AIP56-IL3 was exchanged by dialysis to Tris-NaCl (AIP56-IL3 in IL3 Buf/Tris-NaCl) and its cleavage activity compared to wild type AIP56 (wt) in Tris/NaCl and AIP56-IL3 refolded against IL3 buffer (AIP56-IL3 in IL3 Buf) in the presence of the indicated incubating buffer. The values are indicated in microliters (µL).

As expected, incubation of ^{35}S-labelled sbp65Rel with AIP56 (in Tris-NaCl) resulted in p65 cleavage (figure 15B). The same result was observed for AIP56-IL3 when refolding was performed by dialysis against Tris-NaCl. However, no cleavage was observed when refolding of AIP56-IL3 was performed by dialysis against IL3 buffer or when IL3 buffer was added to AIP56 as control reaction, suggesting that this buffer is inhibiting AIP56's enzymatic activity. The inhibitory effect of the IL3 buffer was confirmed, as exchanging the IL3 buffer to Tris-NaCl by dialysis resulted in p65 cleavage by AIP56-IL3, but only when Tris-NaCl is used as incubating buffer (figure 15C, lane 9). The cleavage activity of wild type AIP56 is also inhibited when IL3 buffer is used as incubating buffer (figure 15C, lane 5). Moreover, when the cleavage activity of wild type AIP56 is tested by mixing IL3 buffer with Tris-NaCl buffer (figure 15C, lane 7 and 8) in the same proportion (4 µL IL3 buffer:12,99 µL Tris-NaCl or 8 µL IL3 buffer:8,99 µL Tris-NaCl) as the one used for testing the cleavage activity of AIP56-IL3, refolded in IL3 buffer (4 µl or 8 µl), in Tris-NaCl (15,5 µL or 11,5 µL) (figure 15C, lane 3 and 6, respectively), its cleavage activity is reduced, when compared with the AIP56(wt) cleavage activity tested with Tris-NaCl only. This suggests that the AIP56-IL3 preparation may not be as stable as that of wild type AIP56 or refolding of the AIP56 domain in the AIP56-IL3 chimera is not absolutely resembling the one of wild type toxin.

Cleavage of NF-κB p65 by the chimeric protein (AIP56-IL3 refolded against Tris-NaCl) was also evaluated using AML cell lysates (figure 16), therefore, directly testing cleavage activity on cellular p65. Three incubation buffers were first tested: Tris-NaCl buffer, lysis buffer and IL-3 buffer (figure 16A). These results are in agreement with those described above for *in vitro* translated p65, given that p65 cleavage was detected only when Tris-NaCl was part of the incubation buffers, and inhibited when IL3 buffer is used. However, enzymatic activity was greatly reduced in the Tris-NaCl buffer when compared to the activity in lysis buffer, in which triton and glycerol are present. Therefore, experiments for testing the effect of different incubating buffers, containing triton and/or

glycerol, on the enzymatic activity of AIP56-IL3 in AML cell lysates were performed (figure 16B). For this purpose, triton and/or glycerol were added to 20mM Tris-200 mM NaCl or 10mM Tris-150 mM NaCl, until a similar condition to that of lysis buffer has been reached. However, in none of the conditions the efficiency of p65 cleavage by AIP56-IL3 was compared to that observed when lysis buffer was used. These results suggested that recombinant AIP56-IL3 is not as stable as wild type AIP56 because, when the components of the incubating buffer are slightly changed, p65 cleavage by AIP56-IL3 is strongly compromised when compared to that of wild type AIP56 (figure 16B).

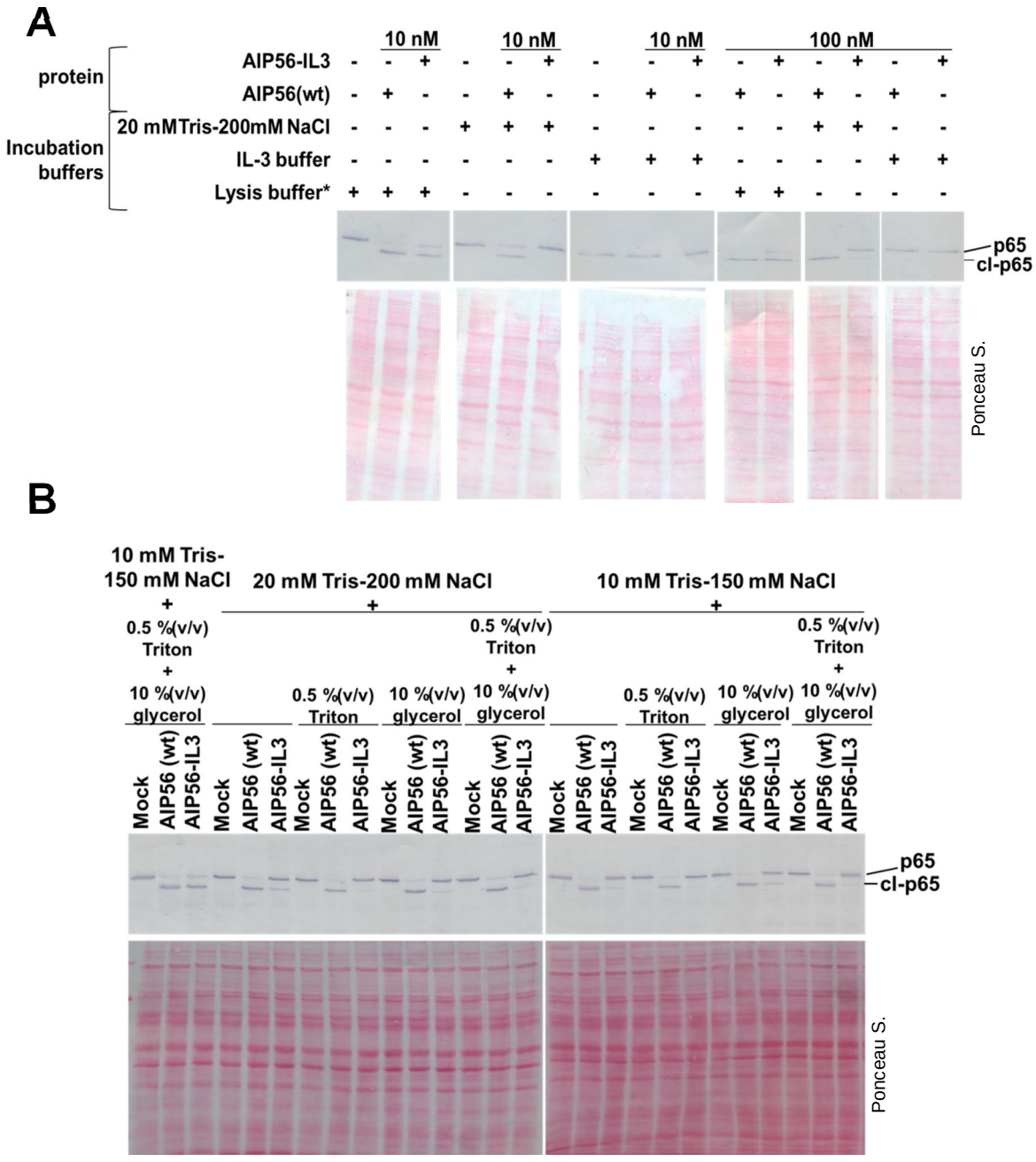

Figure 16. Cleavage activity on cellular NF-κB p65. The lysates were incubated for 2 hours at 22°C with indicated protein and cleavage assessed by Western blotting **A)** Incubation of AML cell lysates with 10 nM or 100 nM of AIP56 or AIP56-IL3 refolded by dialysis against Tris-NaCl in the presence of the indicated incubation buffers. B) Incubation of AML cell lysates

with 100 nM of AIP56 or AIP56-IL3 refolded by dialysis against Tris-NaCl, in the presence of the indicated incubation buffers. Incubation of AML cell lysates without protein and with indicated buffer (Mock) was used as control. The corresponding Ponceau is shown.
Lysis buffer: 10 mM Tris-HCl pH 8.0, 0.5 % (v/v) Triton X-100, 150 mM NaCl and 10 % (v/v) glycerol. The position of the full length (p65) and cleaved p65 (cl-p65) are indicated.

Considering that direct refolding by dialysis against Tris-NaCl was the faster and cheaper process this condition was chosen for proceeding to large-scale production of active AIP56-IL3. However, the refolding process was further improved by testing the initial concentration of insoluble protein that would render the best recovering of soluble protein. For this purpose, different initial protein concentration was tested, ranging from 0.1 to 1 mg/mL. Results showed that the best yield (figure 17A) and p65 cleavage efficiency (Fig. 17B) was obtained when the starting concentration of unfolded AIP56-IL3 was 0.2 mg/mL.

The soluble refolded AIP56-IL3 had a purity >81% (figure 2S in Annex). Functional assays were performed with no further purification procedures.

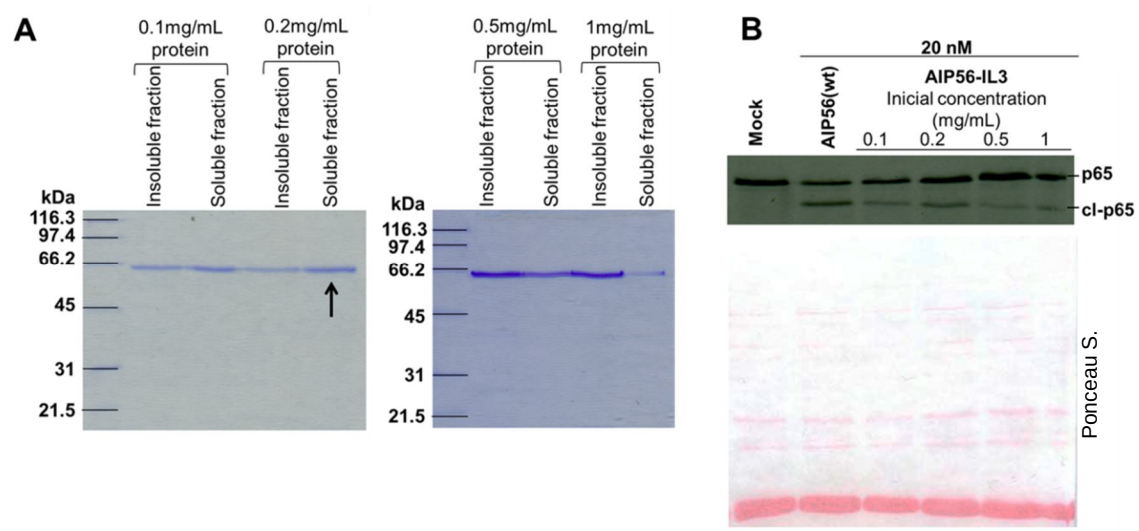

Figure 17. Improvement of the refolding process by dialysis against Tris-NaCl. A) Different concentrations of unfolded AIP56-IL3 (top of the gel) were dialyzed against Tris-NaCl. Insoluble and soluble fractions were analyzed by Coomassie-blue stained 12 % acrylamide SDS-PAGE. The best condition for obtaining the higher proportion of soluble AIP56-IL3 is indicated by an arrow. B) Catalytic activity of AIP56-IL3. The p65 cleavage activity from AIP56-IL3, resulting from the refolding from different initial protein concentrations (indicated in figure), was analyzed *in vitro* by incubation with translated ^{35}S-labeled sbp65Rel. Wild type AIP56 was used as control. The mixture was incubated for 2 hours at 22°C with 20 nM of the indicated protein and p65 cleavage assessed by autoradiography. Position of full length (p65) and cleaved p65 (cl-p65) are indicated. The corresponding membrane stained with Ponceau is shown below.

In conclusion, the first part of this work, which included the production of chimeric proteins, was successfully achieved for AIP56-IL3. As AIP56-IL3 was recovered in insoluble state, refolding processes were tested, with two of them, direct dialysis against Tris-NaCl and dialysis against IL3 buffer followed by buffer exchange by dialysis against

Tris-NaCl, resulting in successful recovering of soluble and active chimeric protein. Taking into account that the chimera has two independent domains, the catalytic and the receptor binding, it is still necessary to validate the correct folding of the IL3 cell receptor-binding domain, used in this chimera. For this purpose, intoxication and internalization assays were developed, as shown below in the second part of this work.

4. AIP56-IL3 did not cleave p65 in AML cell lines

To elucidate whether replacement of AIP56's putative receptor binding domain by particular moieties leads to toxicity in specific cell types, cleavage of p65 was used as readout for the arrival of the chimera or chimera's catalytic domain into the cytosolic compartment. Induction of apoptosis was also evaluated, as cleavage of p65 by AIP56 was shown to trigger apoptotic cell death in macrophages.

Given that several cancer cells possess their own membrane signature and often some of those molecules are overexpressed, the biological signal can be easily amplified and these characteristics harnessed to direct molecules of interest. Taking into account that AML cells overexpress IL-3 receptor at cell surface [108] and the binding of IL3 to its receptor causes rapid receptor-mediated endocytosis [68], the detection of proteolytic activity towards p65, located in the cytosol, will indicate the internalization and successful delivery of AIP56-IL3 and/or its catalytic domain into AML cell cytosol.

For this purpose, and given that different cell lines express different receptor levels, three AML cell lines were used. Cells were incubated continuously with AIP56 or AIP56-IL3 proteins, at different times and p65 proteolysis assessed by Western blotting. Different doses were tested, from doses enough to observe depletion of p65 and death in fish macrophages [74] to higher doses, to have into account possible lower catalytic activity of the refolded chimera when compared to wild type toxin. Untreated and mock-treated cells were used as control.

As shown in figure 18A, no changes in p65 level was observed upon incubation with either protein at any of the doses and incubating times tested, independently of the cell line used. Consequently, no apoptosis signs were also observed (not shown). Given the constitutively active expression of NF-κB p65 in blasts of acute myeloid leukemia [109, 110], it could have happened that decreasing of p65 band due to cleavage by AIP56 and/or AIP56-IL3 was not observed because of the p65 overexpression in these cells. The absence of p65-cleavage fragment (cl-p65) could have also been due to its degradation by the proteasome machinery, as already shown to occur when macrophages are incubated with AIP56 (in press). To discard the above assumptions, KG-1 cells were incubated with high doses of AIP56 and AIP56-IL3 in the presence of the proteasomal

inhibitor MG132. However, no cl-p65 was detected, confirming that indeed p65 was not cleaved by any of the proteins tested (figure 18B).

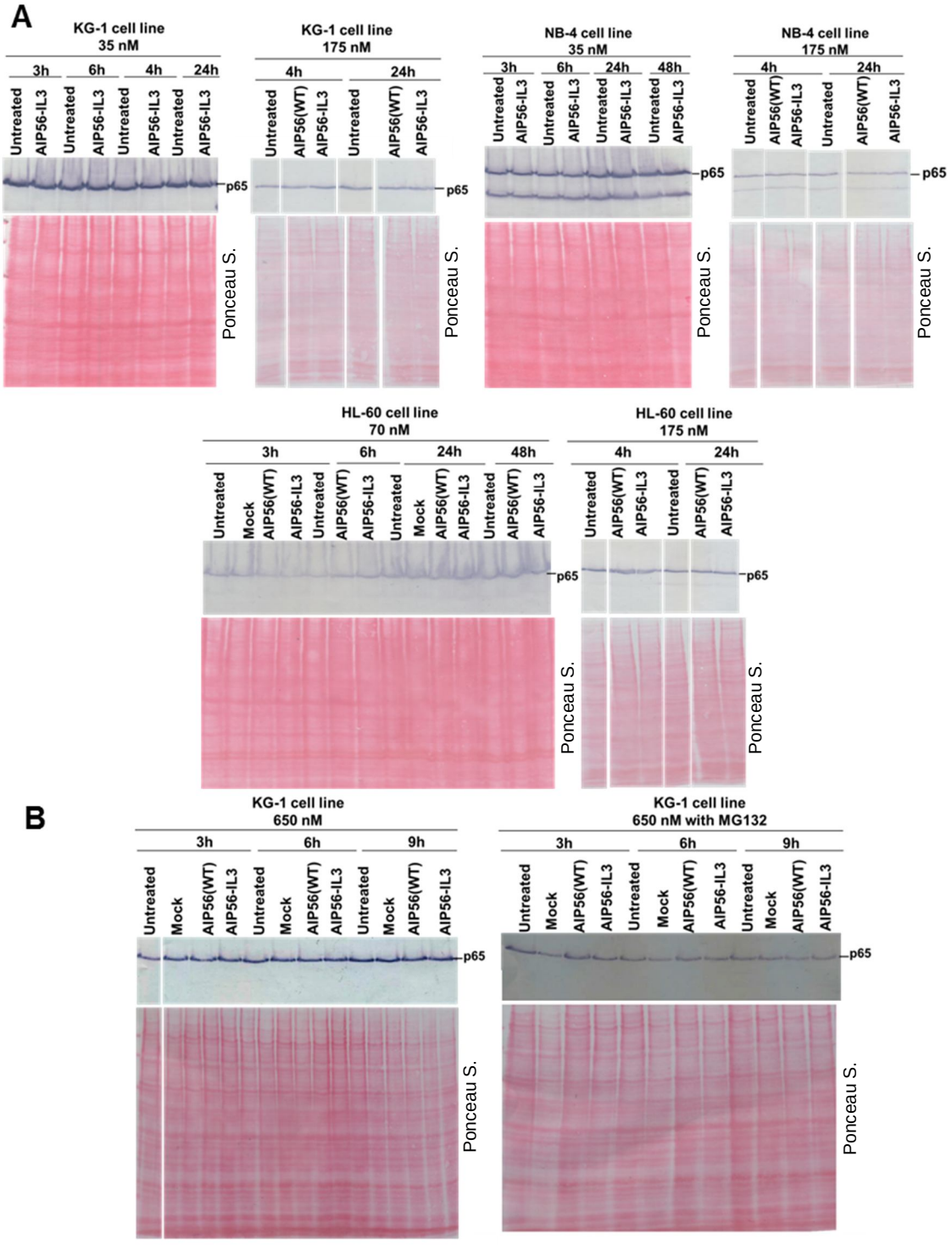

Figure 18. Catalytic activity of AIP56-IL3 in AML cells. A) NF-κB p65 was not cleaved by AIP56-IL3. Different AML cell lines (top of the figure) were incubated with AIP56-IL3 for the indicated time and p65 cleavage detected by Western blotting. Protein concentrations are indicated in each respective figure. Untreated cells, mock treated cells and cells incubated with AIP56 (in some experiments) were used as controls. B) Cells were incubated with high doses of AIP56 and AIP56-IL3 in the presence of the proteasomal inhibitor MG132.

These results suggest that the catalytic portion of both AIP56 and AIP56-IL3 was unable to reach the cytosol. Therefore, internalization assays were attempted to check whether the lack of p65 cleavage was due to low number or lack of cell associated specific receptor, for AIP56, or incorrect folding of IL3 domain, with concomitant inhibition of binding, or inhibition of translocation of the catalytic domain, in AIP56-IL3.

However, although several attempts have been done to properly develop a suitable protocol for testing internalization of AIP56-IL3 in AML cells, no p65 cleavage was observed (see annex section figure 4S). Nevertheless, the results obtained may have been compromised, mostly by lacking of a proper antibody for detecting AIP56-IL3. Actually, AIP56 internalization assays in macrophages have been previously performed with success, but the AIP56 used on those tests contained a fused V5 tag that allowed the use of an anti-V5 antibody for detecting internalized AIP56. Lack of a good antibody becomes even more detrimental due to the loss of cells in conditions treated with pronase, which use is essential in internalization tests as it removes membrane bound toxin, therefore, allowing detection of only internalized toxin.

5. Delivered of AIP56 catalytic domain into cell cytosol by the LF/PA delivery system resulted in p65 cleavage but not apoptosis

Given that the internalization assays precluded any conclusion on whether AIP56-IL3 toxicity in AML cells failed due to improper folding of the IL3 domain within AIP56-IL3, therefore inhibiting binding of AIP56-IL3 to AML cells, or due to inability to translocate the enzymatic domain into the cell cytosol, an alternative approach for delivering the AIP56 catalytic domain into the cell cytosol of AML cells was considered. Knowing the success of anthrax LF/PA system in delivering the catalytic domain of several toxins, such as diphtheria, shiga and pseudomonas [74, 111-113], and that this system has been used successfully for delivering the catalytic domain of AIP56 into the cell cytosol of fish and mouse macrophages [74], it become an obvious approach for delivering AIP56 catalytic domain into AML cells and evaluating its performance in inducing apoptosis.

In this work, an AIP56 catalytic domain-LF chimeric protein [74], consisting on LF N-terminal region (LFN) fused with AIP56's N-terminal enzymatic region (LF^{11-263}·$AIP56^{1-261}$), was used together with PA. In order to evaluate the delivery of AIP56 into the cytosol of AML cells, cleavage of NF-κB p65 was assessed by Western blotting after incubation of AML cells with LF-AIP56 in the presence of PA. Apoptosis was evaluated by morphologic analysis.

Preliminary results (figure 19), showed cleavage of p65 in cells incubated with LF^{11-263}·$AIP56^{1-261}$, although without depletion, indicating efficient delivery of AIP56

catalytic domain into these cells. In opposition, in cells incubated with AIP56 or in control conditions no cleavage was observed. Nevertheless, the observed level of p65 cleavage did not result in apoptogenic activity (not shown).

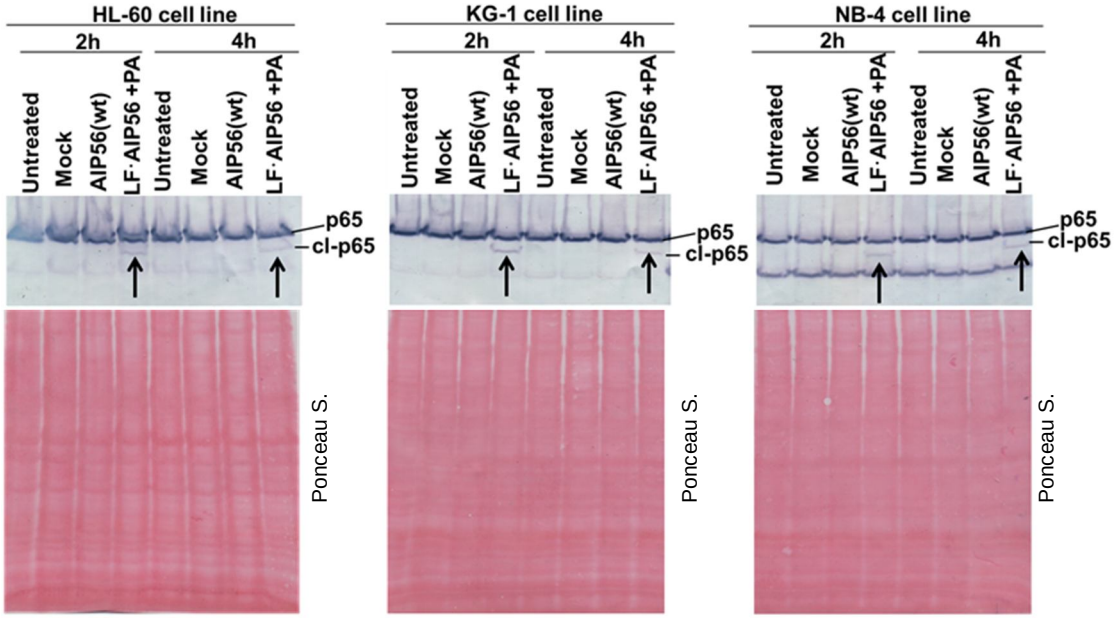

Figure 19. Delivery of the AIP56 N-terminal catalytic region into the AML cell cytosol, using the *Bacilus anthracis* LF/PA system. Different AML cell lines (indicated on top of the figure) were incubated for 2 or 4 h at 37°C with 20 nM LF^{11-263}.$AIP56^{1-261}$ in the presence of 10 nM PA or 100 nM (5.6 µg/mL) AIP56. Mock treated cells with Tris-NaCl were used as control. Cleavage of the NF-κB p65 was detected by Western blotting. The position of the full length (p65) and cleaved p65 (cl-p65) are indicated.

The low p65 cleavage may have been due to loss of some activity of PA because it was not freshly produced for this experiment and have suffered successive cycles of freezing and thawing. This has been supported by using a freshly made batch of PA in the second experiment, using HL-60 cells, because cleavage of p65 was significantly increased (figure 20). However, apoptosis was still not induced.

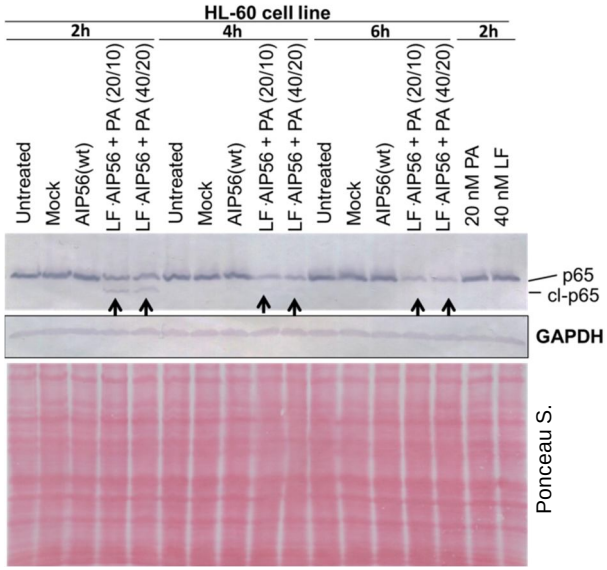

Figure20. Delivery of the AIP56 N-terminal catalytic region into the AML cell cytosol, using the *Bacilus anthracis* LF/PA system, with new batch of PA. Hl-60 cell line was incubated for 2, 4 or 6 h at 37°C with 20 nM LF^{11-263}.$AIP56^{1-261}$ in the presence of 10 nM PA (indicated as 20/10) or 40 nM LF^{11-263}.$AIP56^{1-261}$ in the presence of 20 nM PA (indicated as 40/20). Mock treated cells with Tris-NaCl and cells incubated with LF or PA were used as control. Cleavage of the NF-κB p65 was detected by Western blotting. The position of the full length (p65) and cleaved p65 (cl-p65) are indicated. Membrane was reprobed with an anti-GAPDH antibody (used as protein loading).

CHAPTER V

DISCUSSION

Over time and following research progression, AB toxins have revealed valuable tools in the study of fundamental cellular functions and are now being investigated for potential applications in the clinical treatment of human diseases like cancer and a variety of autoimmune disorders. Examples of these toxins most often used as tools include diphtheria, anthrax toxins and PE [64].

To this end, toxin-based cell delivery systems avail the structure-function of AB toxins, which are divided into distinct domains, as well as its extremely potency, leading to the use of these toxins as vectors to bring other molecules into cells. The common strategy to design these chimeric proteins involves the combination of the targeting moiety that confers target specificity to the chimeric protein and tropism to specific cells, with a potent effector molecule, the toxin. Thus, this fusion occurs between two independent functional domains, which are, biological and individually, often found in distinct biological systems, performing their activities under specific requirements. One example of this approach is the chimeric protein constructed and produced in this work. This chimera is composed of human IL-3, acting as the targeting moiety, linked to the catalytic domain of AIP56 toxin via molecular biology strategies. AIP56 is produced by *Photobacterium damselae piscicida*, a gram-negative bacterium [70], which infection affects several salt warm water fish species, including sea bass. AIP56 shares the same salt requirements of its producing bacterium [114] and the stability of this toxin is promoted by halophilic environments.

The expression of the majority of heterologous proteins is performed in *E. Coli*, a widely used system that lacks post-translational modification machinery. This fact, alongside with the distinct nature and stability requirements of the moieties, present the most difficult challenges to modern biotechnology for production of soluble and stable chimeric proteins. However, several efforts have been attempted, given that the expression in soluble conditions is paramount for the expressed protein to be biologically active [115]. Although a number of general and protein-specific techniques are available, their effectiveness varies widely. Several strategies to try to identify experimental set-ups leading to the production of soluble proteins include molecular biology techniques, as well as manipulation of the culture environment [116]. In terms of bacterial expression, the system used, bacterial strain, the use of specific additives, concentration of IPTG or temperature are some of the conditions commonly tested [116, 117].

In this work, the choice of the expression conditions in *E. Coli* for obtaining soluble protein was based in previous experience in successful production of soluble AIP56. Nevertheless, regarding the different nature of this chimera, an initial screening of induction temperatures was performed. In all the conditions tested only insoluble protein was obtained. Given the lower production at 22ºC and the fact that the production at 30

and 37ºC was not significant different, induction at the intermediate temperature of 30ºC was selected to proceed.

As a way to find a solution for the solubility issue found earlier, the second attempt to recover soluble protein was made by a lysis buffer screening. In this rescue method, it is hypothesized that a significant fraction of protein is not found in inclusion bodies but rather expressed as soluble protein aggregate after lysis [99, 118]. Thus, the addition of specific additives, that have been shown to increase the stability of proteins *in vivo*, during cell lysis, can prevent the partially unfolded protein aggregation from occurring or leading to the properly folded and non-aggregated state, by acting as chemical chaperones, and consequently aiding in its recovery in the soluble fraction [99, 119]. Overall, some stabilizing properties include: reduction of protein-protein interaction, stabilization of intra-molecular bond or thermally stabilization of proteins [99, 120]. Here, eleven additives known by these properties were tested. However, all of them failed in preventing protein aggregation. Even though several general and protein-specific methods are available for obtaining solubility of expressed proteins in *E. coli*, the proteins, in this system, are often expressed as insoluble aggregates of folding intermediates known as inclusion bodies (IBs) [115]. For AIP56-IL3 chimera, the presence of two disulfide bonds in its structure [74, 121], can contribute widely to the production of insoluble protein observed, due to the bacterial cytoplasmic reducing environment, which is unfavorable for the formation of disulfide bonds, leading often to aggregation of unfolded proteins into IBs [122, 123]. Accordingly, chimeras composed of diphtheria toxin fused IL-3, reported in literature, are obtained by refolding process [17, 97, 100, 106]. Considering this problem, modification of the cytoplasmic reducing environment and/or co-expression of protein chaperones could be used as solving strategies [122, 124].

Other current protocols are available that describe various strategies for the conversion of inactive protein, expressed as insoluble inclusion bodies, into a soluble and active fraction [125]. A common strategy used consists in the solubilization and refolding of inclusion body into an active conformation. However, although considerable progress in the last years has been made for efficient refolding of proteins, this process is not straightforward because specific folding conditions differ greatly from protein to protein [115]. It is not possible to predict which compound will facilitate folding of a particular protein, or whether more than one is required for this process [116], requiring an extensive trial-and-error approach. In case of chimeric proteins, an additive can promote the refolding of one moiety but not the other, representing an instability factor, affecting the overall stability of the protein and therefore its functional activity. The major drawbacks during the refolding are the reduced recovery, usually lower than 10% [115], the requirement for rigorous optimization of refolding conditions for each target protein, and

the possibility that the re-solubilization procedure could affect the activity of the refolded protein [125]. Thus, in many cases it may be difficult and expensive to obtain a soluble functional protein. This way, exploiting the production of recombinant proteins in a soluble form using other methodologies, other than *in vitro* refolding procedures, still remains a preferable alternative [116, 125]. Nevertheless, this may be the only way of obtaining the protein of interest in the biologically active state.

Given that we were confronted by the solubility problem, the refolding approach was performed in an attempt to obtain active protein. As mentioned earlier, this process is characterized by a trial-and-error approach and must be done on a case-by case basis. In the work here presented, different refolding buffers as well as refolding methodologies were tested. The starting point for this test was based in previous successful experience in obtaining soluble AIP56 and AIP56 derivatives (e.g., mutants and truncated forms) using this methodology. Combinations of techniques as well as refolding buffers were tested. Thus, the AIP56-IL3 was produced and subjected to refolding by dialysis against Tris-NaCl. The correct folding of the AIP56 domain in this chimera was demonstrated by the ability of AIP56-IL3 to cleave both ^{35}S-labelled sb p65Rel domain and p65 in AML cell lysates, although the weaker cleavage activity of AIP56-IL3 towards p65 in AML cell lysates, when compared to that of wild type AIP56, suggest that stability of the recombinant chimera was not yet completely achieved.

AIP56 is classified as a single chain AB toxin. Its known main action is against fish neutrophils and macrophages by cleavage of the p65 subunit of NF-κB within its Rel homology domain, triggering cell apoptosis [74]. Recent data showed also that it is able to display the same toxicity in mouse macrophages (*in press*). The fact that its action is towards NF-κB, a transcription factor with pivotal biological role in immune responses and involved in several disorders such as cancer and inflammatory disease [82], makes this toxin a potential and attractive tool for treating diseases where overexpression of NF-κB is reported. Actually, other AB toxins, such as DT or anthrax, are successfully used in the targeting of distinct molecules [64, 113]. The replacement of domains, promoting specific targeting, allowed by distinct structure-function organization (characteristic of these toxins) is a concept widely exploited in therapy. In AIP56 toxin, besides the two majors domains that characterize this toxin (A and B domains), the existence of a putative region/subdomain within the C-terminal B domain that may correspond to a receptor binding region, raised the possibility of directing AIP56 to cells other than phagocytes by replacement of this putative region. For this purpose, and aiming to direct the toxin to specific cancer cells, it was considered the replacement of AIP56 putative receptor binding region by ligands whose membrane receptors are overexpressed in cancer cells. Membrane signing as well as membrane receptors overexpression are the hallmark of a

cancer cell, and are becoming increasingly important in cancer cell therapy, being harnessed as targets of the delivery of drugs [126]. Given this overexpression, the biological signal is often amplified.

In this work, the putative receptor binding domain was successfully replaced by interleukin-3, whose receptor (IL-3R) is over-expressed at membrane of Acute Myeloid Leukemia (AML) cells [67, 127] and the toxicity induced by this chimera analyzed by detection of cleavage of NF-κB p65 subunit, localized in the cytosol, as indicative of enzymatic activity and arrival of AIP56-IL3 or AIP56 domain into the cytosolic compartment. Moreover, it was also analyzed of its ability to trigger apoptosis in these cells.

As an AB toxin, the AIP56 intoxication process is characterized by four main steps: binding to cell receptor, internalization, translocation and intracellular effect. The detection of the proteolytic activity towards p65 will indicate that, necessarily, the chimera AIP56-IL3 successfully completed this route composed by the 4 steps above mentioned.

In this work, it was not observed cleavage of p65 when AIP56-IL3 was incubated with AML cells, in none of the concentrations and time tested. Accordingly, signs of apoptosis were not observed in these cells. Given that in the present work it was shown that correct folding of the AIP56 domain was succeeded, as supported by p65 cleavage upon incubation of AIP56-IL3 with *in vitro* translated p65 and cellular p65 from AML cell lysates, the absence of p65 cleavage when the chimera was incubated with living cells could have been due to: (i) unfolding of the IL3 domain, leading to failure in the first step of the intoxication, i.e., binding to the cell; or (ii) failure in the translocation process. The correct fold of the binding domain is pivotal for recognition of its receptor which then allows internalization to occur. It is known that the binding of IL3 to its receptor causes rapid receptor-mediated endocytosis [68]. Therefore, in normal situations where unfolding is not a limitation, the binding and subsequently internalization of the chimera should occur. Despite all efforts, the verification of correct folding of IL3 domain through internalization assays, where detection of AIP56 inside cell is indicative that the toxin has entered, was unsuccessful. The low sensibility of the antibody used, the demand of this protocol, namely, the need to control the temperature during the experiment, the harshness of pronase as well as DMSF to the cells, leading to its death when it is not well controlled, and the intracellular detection of the protein exogenously added, requiring well done washes, were some of the limitations found. Alternatively, detection of phosphorylation in specific molecules involved in downstream pathways initiated upon IL-3 stimulation, such as in the MAPK and phosphatidylinositol 3-kinase (PI-3K)/AKT pathway [128, 129], proliferation bioassay of TF-1 cells (a factor-dependent human erythroleukemic cell line) [130], or detection of binding by ^{125}I-labeled IL-3, could be

alternatives to assess binding of IL-3 to its receptor and supportive of its correct folding. Perhaps relevant, is the fact that the refolding process selected in the present work, i.e. direct dialysis against Tris-NaCl, may have prevented proper folding of IL-3. As mentioned before, active chimeric proteins composed of DT fused to IL-3 have been obtained through refolding process [17, 97, 100, 106]. However, in this case, refolding was first done by dialysis against IL-3 buffer before buffer exchange to Tris-NaCl during the purification process. Despite the same refolding process has been also used for AIP56-IL3 during small-scale testing, chimeric protein obtained this way remain to be tested with AML cells. Alternatively, the fusion of AIP56 with IL-3 may simply compromise the correct folding of IL-3 or the translocation process precluded, either due to incorrect folding or rigidity of the translocation region, or because the boundaries of the putative AIP56 binding region, replaced in the chimera by IL-3, may have been wrongly defined and, consequently, some amino acid residues, important for translocation, removed. In this respect, it should be noted that AIP56's putative binding region has been defined based on limited proteolysis experiments and bioinformatics analysis of AIP56's secondary structure, and, therefore, exact boundaries of the AIP56 binding/translocation regions are not yet established. Regarding the possible rigidity of the translocation region, this may arise from the fusion of the two independent molecules, AIP56 toxin and IL-3, which can produce a chimera with considerable structural rigidity, unable to undergo essential conformational changes for insertion into the membrane and, therefore, compromising the translocation process. Indeed, similarly to other single chain toxins, such as DT, AIP56 needs to suffer conformational changes, triggered by acidic pH within endosomes, which allow its translocation into the cell cytosol by a not yet defined mechanism. In case of DT, a well understood single chain toxin, it is known that endosome acidification triggers a conformational change of the B subunit, facilitating insertion of the translocation domain into the membrane, which forms a cation selective pore. At low pH, the catalytic subunit also unfolds, becoming hydrophobic, and translocating across the formed channel to the cytosol [54]. Another aspect to consider is that AIP56-IL3 was produced with an additional N-terminal tag composed of six histidines and a TEV cleavable site that was not removed during the purification process. Thus, it cannot be disregarded that this tag can somehow prevent or obstruct insertion and translocation across the membrane. Alternatively, it may happen that, in contrast to other AB toxins, the full length toxin is required for the process to occur. Finally, it is reported that some AB toxins, such as DT and the group of binary toxins, such as anthrax toxin, require host cell chaperones or others cytosolic factors to help in both translocation and refolding of the A domain [37, 131-133]. Given that the molecular mechanisms and contributors for these processes are still unknown for AIP56, and, so far, it has been shown that AIP56 only targets neutrophils and macrophages, the

lack of some promoters in AML cells or the distinct endosomal membrane composition, may also have to be taken into account. Indeed, in the present study, no toxicity was observed upon incubation of AML cells with AIP56, strongly supporting the absence of a specific receptor for AIP56 in these cells. However, as the internalization assays did not work properly, it cannot be discarded that the intoxication process has failed due to lacking of components in AML cells other than the membrane receptor.

Considering the above mentioned limitations, a parallel and complementary experiment aiming at delivering the AIP56 catalytic domain into AML cells by an alternative system was considered. For this purpose, the choice fell on the anthrax LF/PA delivery system, as it was previously successfully used for AIP56 [74]. Anthrax toxin, secreted by *Bacillus anthracis,* is composed by a B unit, called protective antigen (PA), which binds to anthrax toxin receptor (ATR) and is responsible for the delivery into cells of two alternative enzymatic A subunits: lethal factor (LF) and/or edema factor (EF). PA binds to ATR and upon cleavage by a furin proteases, releases a smaller fragment (PA_{20}) extracellularly, while the remaining fragment (PA_{63}) begins the assembly of the toxin complex by self-association, resulting in formation of a heptameric or octameric prepore that finally bind up to a maximum of three or four molecules of LF or EF, respectively. Subsequently, occurs endocytosis and trafficking of this complex to the acid endosomal compartment. Low pH triggers a conformational change in PA, leading to formation of pores by its insertion into the endosome membrane. This way, the translocation of LP or EF to cytosol is promoted [134]. PA is a central component of this system because it mediates the entry of LF or EF into cells but it can also translocate chimeric proteins containing PA-binding determinants. Moreover, the PA's receptor ATR is present in most cell types [134-136] , allowing the wide use of this system as vehicle to delivery proteins into the cell cytosol. It has been demonstrated that the first 263 amino acids of LF, corresponding to the N-terminal, are the minimal region required to allow recognition and binding to PA and subsequently uptake into cell of fused polypeptides [111]. Promising results were obtained when the anthrax LF/PA delivery system was used to deliver the AIP56 catalytic domain into AML cells because cleavage of p65 was observed, showing that, when inside these cells, AIP56 is able to perform its enzymatic activity. However, apoptosis could not be observed in the conditions tested. Three main reasons may account to this circumstance: (i) low expression level of the ATR in AML cells, resulting in insufficient amounts of internalized toxin for causing p65 depletion; indeed, although anthrax toxin is able to enter almost any cell type, its major target are professional phagocytes [134-136]; (ii) need for longer incubation periods; and/or (iii) need of pro-apoptotic co-stimulatory stimuli.

CHAPTER VI

FINAL REMARKS AND FUTURE PERSPECTIVES

In this work, studies on AIP56 toxin were mainly focused on directing it to cells other than macrophages through protein ligands that promote specific targeting. Its ability to induce toxicity in specific cells was evaluated.

The replacement of the putative receptor binding domain by interleukin-3 was performed, yielding AIP56-IL3 chimeric protein. NF-κB p65 cleavage and its ability to induce cell death were assessed in AML cells. Cleavage of p65 by this chimera was not observed in AML cells. Given the limitations found during the course of the project it remains to be elucidated whether the absence of p65 cleavage in living cells resulted from incorrect folding of the AIP56-IL3 chimera. Taking into account the success of the chimera DT-IL3, obtained by refolding trough a two-step dialysis protocol, it should be considered carrying out experiments on AML cells with AIP56-IL3 chimera obtained according to the procedure used for the refolding of DT-IL3. Alternatively, other receptor binding moieties may be considered for replacing the AIP56 binding region, e.g., single chain antibodies towards IL-3 receptor [137]. However, finding the precise boundaries of AIP56's receptor binding region would be greatly helpful.

Finally, using the anthrax LF/PA delivering system for delivering AIP56 into AML cells, allow exploring the effect of targeting p65 into these cells under different circumstances and conclude about the feasibility of using AIP56 catalytic domain as biomedical tool.

CHAPTER VII

REFERENCES

1. Foss, F.M., *Interleukin-2 fusion toxin: targeted therapy for cutaneous T cell lymphoma.* Ann N Y Acad Sci, 2001. **941**: p. 166-76.
2. Figgitt, D.P., H.M. Lamb, and K.L. Goa, *Denileukin diftitox.* Am J Clin Dermatol, 2000. **1**(1): p. 67-72.
3. Puri, R.K., *Development of a recombinant interleukin-4-Pseudomonas exotoxin for therapy of glioblastoma.* Toxicol Pathol, 1999. **27**(1): p. 53-7.
4. Leppla, S.H., *Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells.* Proc Natl Acad Sci U S A, 1982. **79**(10): p. 3162-6.
5. Honjo, T., Y. Nishizuka, and O. Hayaishi, *Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis.* J Biol Chem, 1968. **243**(12): p. 3553-5.
6. Kioi, M., S. Seetharam, and R.K. Puri, *Targeting IL-13Ralpha2-positive cancer with a novel recombinant immunotoxin composed of a single-chain antibody and mutated Pseudomonas exotoxin.* Mol Cancer Ther, 2008. **7**(6): p. 1579-87.
7. Cassel, D. and Z. Selinger, *Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site.* Proc Natl Acad Sci U S A, 1977. **74**(8): p. 3307-11.
8. Potala, S. and R.S. Verma, *Modified DT-IL2 fusion toxin targeting uniquely IL2Ralpha expressing leukemia cell lines - Construction and characterization.* J Biotechnol, 2010. **148**(2-3): p. 147-55.
9. Ugo Testa, R.R., Mauro Biffoni, Daniela Diverio, Francesco Lo-Coco, Robin Foà, Cesare Peschle, and Arthur E. Frankel, *Diphtheria toxin fused to variant human interleukin-3 induces cytotoxicity of blasts from patients with acute myeloid leukemia according to the level of interleukin-3 receptor expression.* Bood, 2005. **106**: p. 2527**–**2529.
10. Vandekerckhove, J., et al., *Botulinum C2 toxin ADP-ribosylates cytoplasmic beta/gamma-actin in arginine 177.* J Biol Chem, 1988. **263**(2): p. 696-700.
11. Gill, D.M. and S.H. Richardson, *Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of Escherichia coli: comparison with cholera toxin.* J Infect Dis, 1980. **141**(1): p. 64-70.
12. Iglewski, B.H., P.V. Liu, and D. Kabat, *Mechanism of action of Pseudomonas aeruginosa exotoxin Aiadenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo.* Infect Immun, 1977. **15**(1): p. 138-44.
13. Just, I., et al., *Glucosylation of Rho proteins by Clostridium difficile toxin B.* Nature, 1995. **375**(6531): p. 500-3.

14. Okajima, F. and M. Ui, *ADP-ribosylation of the specific membrane protein by islet-activating protein, pertussis toxin, associated with inhibition of a chemotactic peptide-induced arachidonate release in neutrophils. A possible role of the toxin substrate in Ca2+-mobilizing biosignaling.* J Biol Chem, 1984. **259**(22): p. 13863-71.
15. Endo, Y., et al., *Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins.* Eur J Biochem, 1988. **171**(1-2): p. 45-50.
16. Nagai, T., et al., *Efficacy of an immunotoxin to folate receptor beta in the intra-articular treatment of antigen-induced arthritis.* Arthritis Research & Therapy, 2012. **14**(3): p. R106.
17. Feuring-Buske, M., et al., *A diphtheria toxin-interleukin 3 fusion protein is cytotoxic to primitive acute myeloid leukemia progenitors but spares normal progenitors.* Cancer Res, 2002. **62**(6): p. 1730-6.
18. Yamada, Y., et al., *Differential effects of denileukin diftitox IL-2 immunotoxin on NK and regulatory T cells in nonhuman primates.* J Immunol, 2012. **188**(12): p. 6063-70.
19. Yoon, D.J., et al., *Intratumoral therapy of glioblastoma multiforme using genetically engineered transferrin for drug delivery.* Cancer Res, 2010. **70**(11): p. 4520-7.
20. Duesbery, N.S., et al., *Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor.* Science, 1998. **280**(5364): p. 734-7.
21. Hill, K.K., et al., *Genetic diversity among Botulinum Neurotoxin-producing clostridial strains.* J Bacteriol, 2007. **189**(3): p. 818-32.
22. Hu, C.C., et al., *Investigation of a plasmid containing a novel immunotoxin VEGF165-PE38 gene for antiangiogenic therapy in a malignant glioma model.* Int J Cancer, 2010. **127**(9): p. 2222-9.
23. von Minckwitz, G., et al., *Phase I clinical study of the recombinant antibody toxin scFv(FRP5)-ETA specific for the ErbB2/HER2 receptor in patients with advanced solid malignomas.* Breast Cancer Res, 2005. **7**(5): p. 1.
24. Kawakami, K., et al., *Targeting interleukin-4 receptors for effective pancreatic cancer therapy.* Cancer Res, 2002. **62**(13): p. 3575-80.
25. Reig, N. and F.G. van der Goot, *Toxins in the Endosomes*, in *Endosomes*2006, Springer New York. p. 145-152.
26. Roux E, Y.A., *Contribution a l´etude de la diphtherie.* Annales de l'Institut Pasteur, 1888. **2**: p. 629-61.
27. Schmitt, C.K., K.C. Meysick, and A.D. O'Brien, *Bacterial toxins: friends or foes?* Emerg Infect Dis, 1999. **5**(2): p. 224-34.

28. *Bacterial Toxins: A Brief Overview*, in *Cholera Toxins*, K. Chaudhuri and S.N. Chatterjee, Editors. 2009, Springer Berlin Heidelberg. p. 5-11.
29. Montecucco, C., E. Papini, and G. Schiavo, *Bacterial protein toxins penetrate cells via a four-step mechanism.* FEBS Lett, 1994. **346**(1): p. 92-8.
30. Balfanz, J., P. Rautenberg, and U. Ullmann, *Molecular Mechanisms of Action of Bacterial Exotoxins.* Zentralblatt für Bakteriologie, 1996. **284**(2**–**3): p. 170-206.
31. Lemichez, E. and J.T. Barbieri, *General aspects and recent advances on bacterial protein toxins.* Cold Spring Harb Perspect Med, 2013. **3**(2).
32. Vega Masignani, M.P., Rino Rappuoli, *Bacterial Toxins*, in *The Prokaryotes* 2006. p. 893-955.
33. Henkel, J.S., M.R. Baldwin, and J.T. Barbieri, *Toxins from bacteria.* Exs, 2010. **100**: p. 1-29.
34. Bohach, G.A., et al., *Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses.* Crit Rev Microbiol, 1990. **17**(4): p. 251-72.
35. Marrack, P. and J. Kappler, *The staphylococcal enterotoxins and their relatives.* Science, 1990. **248**(4959).
36. Miethke, T., et al., *T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor.* J Exp Med, 1992. **175**(1): p. 91-8.
37. Odumosu, O., et al., *AB toxins: a paradigm switch from deadly to desirable.* Toxins, 2010. **2**(7): p. 1612-45.
38. Geny, B. and M.R. Popoff, *Bacterial protein toxins and lipids: pore formation or toxin entry into cells.* Biol Cell, 2006. **98**(11): p. 667-78.
39. Falnes, P.O. and K. Sandvig, *Penetration of protein toxins into cells.* Curr Opin Cell Biol, 2000. **12**(4): p. 407-13.
40. Beddoe, T., et al., *Structure, biological functions and applications of the AB5 toxins.* Trends Biochem Sci, 2010. **35**(7): p. 411-8.
41. Nesic, D., Y. Hsu, and C.E. Stebbins, *Assembly and function of a bacterial genotoxin.* Nature, 2004. **429**(6990): p. 429-33.
42. Yamada, T., et al., *Variation of loop sequence alters stability of cytolethal distending toxin (CDT): crystal structure of CDT from Actinobacillus actinomycetemcomitans.* Protein Sci, 2006. **15**(2): p. 362-72.
43. Bezine, E., J. Vignard, and G. Mirey, *The cytolethal distending toxin effects on Mammalian cells: a DNA damage perspective.* Cells, 2014. **3**(2): p. 592-615.
44. Lina Guerra, X.C.-B., Riccardo Guidi, Teresa Frisan, *The Biology of the Cytolethal Distending Toxins.* Toxins, 2011. **3**: p. 172-190.

45. Pickett, C.L., et al., *Cloning, sequencing, and expression of the Escherichia coli cytolethal distending toxin genes.* Infect Immun, 1994. **62**(3): p. 1046-51.
46. Song, J., X. Gao, and J.E. Galan, *Structure and function of the Salmonella Typhi chimaeric A(2)B(5) typhoid toxin.* Nature, 2013. **499**(7458): p. 350-4.
47. Simon, N.C., K. Aktories, and J.T. Barbieri, *Novel bacterial ADP-ribosylating toxins: structure and function.* Nat Rev Microbiol, 2014. **12**(9): p. 599-611.
48. Leppla, V.M.G.a.S.H., *Proteolytic activation of bacterial toxins: role of bacterial and host cell proteases.* Infect Immun, 1994. **62(2)**: p. 333**–**340.
49. Tsuneoka M, N.K., Hatsuzawa K, Komada M, Kitamura N, Mekada E, *Evidence for involvement of furin in cleavage and activation of diphtheria toxin.* J Biol Chem, 1993. **268**: p. 26461-26465.
50. Gill DM, D.L., *Observations on the structure of diphtheria toxin.* J Biol Chem, 1971. **246**: p. 1485-1491.
51. Geny, B. and M.R. Popoff, *Bacterial protein toxins and lipids: role in toxin targeting and activity.* Biol Cell, 2006. **98**(11): p. 633-51.
52. Sandvig, K. and B. van Deurs, *Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives.* Embo J, 2000. **19**(22): p. 5943-50.
53. T R Hirst, J.S., J B Kaper, S J Hardy, and J Holmgren, *Mechanism of toxin secretion by Vibrio cholerae investigated in strains harboring plasmids that encode heat-labile enterotoxins of Escherichia coli.* Proc Natl Acad Sci U S A., 1984. **81(24)**: p. 7752**–**7756.
54. Rosconi, M.P., G. Zhao, and E. London, *Analyzing topography of membrane-inserted diphtheria toxin T domain using BODIPY-streptavidin: at low pH, helices 8 and 9 form a transmembrane hairpin but helices 5-7 form stable nonclassical inserted segments on the cis side of the bilayer.* Biochemistry, 2004. **43**(28): p. 9127-39.
55. Liu, S., M. Moayeri, and S.H. Leppla, *Anthrax lethal and edema toxins in anthrax pathogenesis.* Trends Microbiol, 2014. **22**(6): p. 317-25.
56. Young JA, C.R., *Anthrax toxin: receptor binding, internalization, pore formation, and translocation.* Annu Rev Biochem, 2007. **76**: p. 243-265.
57. Kintzer AF, S.H., Tang, II, Williams ER, Krantz BA, *Anthrax toxin receptor drives protective antigen oligomerization and stabilizes the heptameric and octameric oligomer by a similar mechanism.* PLoS ONE, 2010. **5**: p. e13888.
58. Abrami, L., et al., *Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process.* J Cell Biol, 2003. **160**(3): p. 321-8.

59. Wernick NLB, C.D.-F., Cho JA, Lencer WI, *Cholera Toxin: An Intracellular Journey into the Cytosol by Way of the Endoplasmic Reticulum.* Toxins, 2010. **2**: p. 310-325.
60. Holmgren, J.S.a.J., *Cholera toxin — A foe & a friend.* Indian J Med Res, 2011. **133(2)**: p. 153**–**163.
61. Sandvig, K., et al., *Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum.* Nature, 1992. **358**(6386): p. 510-2.
62. Popoff, J.E.A.a.M.R., *Chapiter 59 - Toxin as tools. In The Comprehensive Sourcebook of Bacterial Protein Toxins (Third Edition).* London: Academic Press, 2006: p. 976.
63. Mathew, M. and R.S. Verma, *Humanized immunotoxins: a new generation of immunotoxins for targeted cancer therapy.* Cancer Sci, 2009. **100**(8): p. 1359-65.
64. Verma, M.J.a.R.S., *Therapeutic targets and recent advances in protein immunotoxins.* Current Opiinion in Microbiology, 2012. **15**: p. 300-309.
65. FitzGerald, A.A.a.D., *Immunotoxins: The Role of the Toxin.* Toxins 2013. **5(8)**: p. 1486-1502.
66. Tatro, J.B., et al., *Interaction of an alpha-melanocyte-stimulating hormone-diphtheria toxin fusion protein with melanotropin receptors in human melanoma metastases.* Cancer Res, 1992. **52**(9): p. 2545-8.
67. Budel, L.M., et al., *Interleukin-3 and granulocyte-monocyte colony-stimulating factor receptors on human acute myelocytic leukemia cells and relationship to the proliferative response.* Blood, 1989. **74**(2): p. 565-71.
68. Nicola, N.A., et al., *Cellular processing of murine colony-stimulating factor (Multi-CSF, GM-CSF, G-CSF) receptors by normal hemopoietic cells and cell lines.* Growth Factors, 1988. **1**(1): p. 41-9.
69. Su, Y., et al., *Characterization of variant diphtheria toxin-interleukin-3 fusion protein, DTIL3K116W, for phase I clinical trials.* Biologicals, 2010. **38**(1): p. 144-9.
70. do Vale, A., et al., *AIP56, a novel plasmid-encoded virulence factor of Photobacterium damselae subsp. piscicida with apoptogenic activity against sea bass macrophages and neutrophils.* Mol Microbiol, 2005. **58**(4): p. 1025-38.
71. do Vale, A., F. Marques, and M.T. Silva, *Apoptosis of sea bass (Dicentrarchus labrax L.) neutrophils and macrophages induced by experimental infection with Photobacterium damselae subsp. piscicida.* Fish Shellfish Immunol, 2003. **15**(2): p. 129-44.
72. Silva, M.T., *Secondary necrosis: The natural outcome of the complete apoptotic program.* FEBS Letters, 2010. **584**(22): p. 4491-4499.

73. Silva, M.T., N.M. Dos Santos, and A. do Vale, *AIP56: a novel bacterial apoptogenic toxin.* Toxins, 2010. **2**(4): p. 905-18.
74. Silva, D.S., et al., *The apoptogenic toxin AIP56 is a metalloprotease A-B toxin that cleaves NF-kappab P65.* PLoS Pathog, 2013. **9**(2): p. 28.
75. Jongeneel, C.V., J. Bouvier, and A. Bairoch, *A unique signature identifies a family of zinc-dependent metallopeptidases.* FEBS Lett, 1989. **242**(2): p. 211-4.
76. Schiavo, G., et al., *Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc.* Embo J, 1992. **11**(10): p. 3577-83.
77. Costa-Ramos, C., et al., *The bacterial exotoxin AIP56 induces fish macrophage and neutrophil apoptosis using mechanisms of the extrinsic and intrinsic pathways.* Fish Shellfish Immunol, 2011. **30**(1): p. 173-81.
78. Baruch, K., et al., *Metalloprotease type III effectors that specifically cleave JNK and NF-kappaB.* Embo J, 2011. **30**(1): p. 221-31.
79. Sen R and B. D., *Multiple nuclear factors interact with the immunoglobulin enhancer sequences.* Cell 1986. **46**: p. 705-716.
80. May, M.J. and S. Ghosh, *Rel/NF-kappa B and I kappa B proteins: an overview.* Semin Cancer Biol, 1997. **8**(2): p. 63-73.
81. Gilmore, T.D., *Introduction to NF-kappaB: players, pathways, perspectives.* Oncogene, 2006. **25**(51): p. 6680-4.
82. Hoesel, B. and J.A. Schmid, *The complexity of NF-kappaB signaling in inflammation and cancer.* Mol Cancer, 2013. **12**(86): p. 1476-4598.
83. Ghosh, M.S.H.a.S., *Signaling to NF-kB.* Genes Dev., 2004. **18**: p. 2195-2224.
84. Lawrence, T., *The nuclear factor NF-kappaB pathway in inflammation.* Cold Spring Harb Perspect Biol, 2009. **1**(6): p. 7.
85. Neish, A.S.a.M.N., *Microbial-induced immunomodulation by targeting the NF-kappaB system.* Trends Microbiol, 2011.
86. Ghosh, A.O.a.S., *The NF-κB Family of Transcription Factors and Its Regulation.* Cold Spring Harb Perspect Biol, 2009.
87. Baeuerle PA, H.T., *Function and activation of NF-k B in the immune system.* Annu Rev Immunol 1994. **12**: p. 141**–**179.
88. Ghosh G, W.V.-F., Huang D-B, Fusco A, *NF-κB regulation: lessons from structures.* Immunol Rev 2012. **246**: p. 36-58.
89. S-C, S., *Non-canonical NF-κB signaling pathway.* Cell Res 2010. **21**: p. 71**–**85.
90. Hayden, M.S. and S. Ghosh, *NF-kappaB, the first quarter-century: remarkable progress and outstanding questions.* Genes Dev, 2012. **26**(3): p. 203-34.

91. Scheidereit, C., *IkappaB kinase complexes: gateways to NF-kappaB activation and transcription.* Oncogene, 2006. **25**(51): p. 6685-705.
92. Hooper, C. *Overview of NFkB signaling*. 2006; Available from: http://www.abcam.com/?pageconfig=resource&rid=11255&pid=10629.
93. Monica L. Guzman, S.J.N., Donna Upchurch, Barry Grimes, Dianna S. Howard, David A. Rizzieri,Selina M. Luger and Craig T. Jordan, *Nuclear factor-kB is constitutively activated in primitive human acute myelogenous leukemia cells.* Blood, 2001 **98**(8).
94. Kucharczak, J., et al, *To be or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis.* Oncogene. **22**: p. 8961-82.
95. Yamamoto, Y.a.R.B.G., *Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer.* J Clin Invest, 2001. **107**: p. 135-42.
96. Haefner, B., *Targeting NF-kappaB in anticancer adjunctive chemotherapy.* Cancer Treat Res, 2006. **130**: p. 219-45.
97. Frankel, A.E., et al., *Characterization of diphtheria fusion proteins targeted to the human interleukin-3 receptor.* Protein Eng, 2000. **13**(8): p. 575-81.
98. Bertani, G., *Lysogeny at mid-twentieth century: P1, P2, and other experimental systems.* J Bacteriol, 2004. **186**(3): p. 595-600.
99. Leibly, D.J., et al., *Stabilizing additives added during cell lysis aid in the solubilization of recombinant proteins.* PLoS ONE, 2012. **7**(12): p. 20.
100. Frankel, A.E., et al., *Diphtheria toxin fused to human interleukin-3 is toxic to blasts from patients with myeloid leukemias.* Leukemia, 2000. **14**(4): p. 576-85.
101. Tenen, D.G., *Disruption of differentiation in human cancer: AML shows the way.* Nat Rev Cancer, 2003. **3**(2): p. 89-101.
102. Birnie, G.D., *The HL60 cell line: a model system for studying human myeloid cell differentiation.* Br J Cancer Suppl, 1988. **9**: p. 41-5.
103. Koeffler, H.P. and D.W. Golde, *Human myeloid leukemia cell lines: a review.* Blood, 1980. **56**(3): p. 344-50.
104. Lanotte, M., et al., *NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3).* Blood, 1991. **77**(5): p. 1080-6.
105. Hacker, G., *The morphology of apoptosis.* Cell Tissue Res, 2000. **301**(1): p. 5-17.
106. Frankel, A.E., et al., *High-level expression and purification of the recombinant diphtheria fusion toxin DTGM for PHASE I clinical trials.* Protein Expr Purif, 1999. **16**(1): p. 190-201.

107. Silva, D.S., et al., *The Apoptogenic Toxin AIP56 Is a Metalloprotease A-B Toxin that Cleaves NF-κb P65.* PLoS Pathog, 2013. **9**(2): p. e1003128.
108. Testa, U., et al., *Elevated expression of IL-3Ralpha in acute myelogenous leukemia is associated with enhanced blast proliferation, increased cellularity, and poor prognosis.* Blood, 2002. **100**(8): p. 2980-8.
109. Bueso-Ramos, C.E., et al., *Expression of constitutively active nuclear-kappa B RelA transcription factor in blasts of acute myeloid leukemia.* Hum Pathol, 2004. **35**(2): p. 246-53.
110. Guzman, M.L., et al., *Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells.* Blood, 2001. **98**(8): p. 2301-7.
111. Arora, N. and S.H. Leppla, *Residues 1-254 of anthrax toxin lethal factor are sufficient to cause cellular uptake of fused polypeptides.* J Biol Chem, 1993. **268**(5): p. 3334-41.
112. Arora, N. and S.H. Leppla, *Fusions of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells.* Infect Immun, 1994. **62**(11): p. 4955-61.
113. Milne, J.C., et al., *Protective antigen-binding domain of anthrax lethal factor mediates translocation of a heterologous protein fused to its amino- or carboxy-terminus.* Mol Microbiol, 1995. **15**(4): p. 661-6.
114. Magarinos, B., et al., *Phenotypic, antigenic, and molecular characterization of Pasteurella piscicida strains isolated from fish.* Appl Environ Microbiol, 1992. **58**(10): p. 3316-22.
115. Ghosh, S., et al., *Method for enhancing solubility of the expressed recombinant proteins in Escherichia coli.* Biotechniques, 2004. **37**(3): p. 422-3.
116. Laura A. Palomares, S.E.-M., and Octavio T. Ramírez, *Production of Recombinant Proteins - Challenges and Solutions*, in *Recombinant Gene Expression*, P.B.a.A. Lorence, Editor 2004. p. 15-51.
117. Einsfeldt, K., et al., *Cloning and expression of protease ClpP from Streptococcus pneumoniae in Escherichia coli: study of the influence of kanamycin and IPTG concentration on cell growth, recombinant protein production and plasmid stability.* Vaccine, 2011. **29**(41): p. 7136-43.
118. Bondos, S.E. and A. Bicknell, *Detection and prevention of protein aggregation before, during, and after purification.* Analytical Biochemistry, 2003. **316**(2): p. 223-231.
119. Ignatova, Z. and L.M. Gierasch, *Inhibition of protein aggregation in vitro and in vivo by a natural osmoprotectant.* Proc Natl Acad Sci U S A, 2006. **103**(36): p. 13357-61.

120. Kaushik, J.K. and R. Bhat, *Thermal Stability of Proteins in Aqueous Polyol Solutions: Role of the Surface Tension of Water in the Stabilizing Effect of Polyols.* The Journal of Physical Chemistry B, 1998. **102**(36): p. 7058-7066.
121. Clark-Lewis, L.E.H., and S B Kent, *Role of disulfide bridges in determining the biological activity of interleukin 3.* Proc Natl Acad Sci U S A., 1988. **85**(21): p. 7897-7901.
122. Kong, B. and G.L. Guo, *Soluble Expression of Disulfide Bond Containing Proteins FGF15 and FGF19 in the Cytoplasm of Escherichia coli.* PLoS ONE, 2014. **9**(1): p. e85890.
123. Tsumoto, K., et al., *Role of arginine in protein refolding, solubilization, and purification.* Biotechnol Prog, 2004. **20**(5): p. 1301-8.
124. Schlieker, C., B. Bukau, and A. Mogk, *Prevention and reversion of protein aggregation by molecular chaperones in the E. coli cytosol: implications for their applicability in biotechnology.* Journal of Biotechnology, 2002. **96**(1): p. 13-21.
125. Sahdev, S., S.K. Khattar, and K.S. Saini, *Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies.* Mol Cell Biochem, 2008. **307**(1-2): p. 249-64.
126. Kampen, K.R., *Membrane proteins: the key players of a cancer cell.* J Membr Biol, 2011. **242**(2): p. 69-74.
127. Munoz, L., et al., *Interleukin-3 receptor alpha chain (CD123) is widely expressed in hematologic malignancies.* Haematologica, 2001. **86**(12): p. 1261-9.
128. Reddy, E.P., et al., *IL-3 signaling and the role of Src kinases, JAKs and STATs: a covert liaison unveiled.* Oncogene, 2000. **19**(21): p. 2532-47.
129. Testa, U., et al., *Interleukin-3 receptor in acute leukemia.* Leukemia, 2004. **18**(2): p. 219-26.
130. Kitamura, T., et al., *Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin.* J Cell Physiol, 1989. **140**(2): p. 323-34.
131. Eva Kaiser, C.K., Katharina Ernst, Carsten Schwan, Michel Popoff, Gunter Fischer, Johannes Buchner, Klaus Aktories and Holger Barth, *Membrane Translocation of Binary Actin-ADP-Ribosylating Toxins from Clostridium difficile and Clostridium perfringens Is Facilitated by Cyclophilin A and Hsp90* Infect Immun., 2011. **79**(10): p. 3913**–**3921.
132. Kaiser, E., et al., *Cyclophilin A facilitates translocation of the Clostridium botulinum C2 toxin across membranes of acidified endosomes into the cytosol of mammalian cells.* Cell Microbiol, 2009. **11**(5): p. 780-95.

133. Haug, G., et al., *The host cell chaperone Hsp90 is essential for translocation of the binary Clostridium botulinum C2 toxin into the cytosol.* J Biol Chem, 2003. **278**(34): p. 32266-74.
134. Ascenzi, P., et al., *Anthrax toxin: a tripartite lethal combination.* FEBS Lett, 2002. **531**(3): p. 384-8.
135. Collier, R.J. and J.A. Young, *Anthrax toxin.* Annu Rev Cell Dev Biol, 2003. **19**: p. 45-70.
136. Friedlander, A.M., *Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process.* J Biol Chem, 1986. **261**(16): p. 7123-6.
137. Jin, L., et al., *Monoclonal Antibody-Mediated Targeting of CD123, IL-3 Receptor α Chain, Eliminates Human Acute Myeloid Leukemic Stem Cells.* Cell Stem Cell, 2009. **5**(1): p. 31-42.
138. Vagenende, V., M.G. Yap, and B.L. Trout, *Mechanisms of protein stabilization and prevention of protein aggregation by glycerol.* Biochemistry, 2009. **48**(46): p. 11084-96.

ANNEX

His-Tag pET28a NdeI TEV cleavage

```
atgggcagcagccatcatcatcatcatcacagcagcggcctggtgccgcgcggcagccatatggagaatctttattttcagagcaac
 M  G  S  S  H  H  H  H  H  H  S  S  G  L  V  P  R  G  S  H  M  E  N  L  Y  F  Q  S  N
aacgataaaccagatgcaagcgatgacaagtacgcagactacgtggtacgtctaggttcggaacatccactaaaccatactcagatc
 N  D  K  P  D  A  S  D  D  K  Y  A  D  Y  V  V  R  L  G  S  E  H  P  L  N  H  T  Q  I
Attgaactttcctctgcagtatcgagggctgtccttcttagttacccaaatataatagaccgatacaccgctgcagcaactgaatat
 I  E  L  S  S  A  V  S  S  A  V  L  L  S  Y  P  N  I  I  D  R  Y  T  A  A  A  T  E  Y
acggtgatcgatgctttatttcattcgcctacctttcgacatatcgtttcttttggtcttcataatcagcaagagaaccttggtcat
 T  V  I  D  A  L  F  H  S  P  T  F  R  H  I  V  S  F  G  L  H  N  Q  Q  E  N  L  G  H
Attcgatatactaatgaatatgaaattaacaataatcgcgaagatgagttctccttagtgagcgaggtaagctacgacgatataaaa
 I  R  Y  T  N  E  Y  E  I  N  N  N  R  E  D  E  F  S  L  V  S  E  V  S  Y  D  D  I  K
Agctctaatgctcagcaagttcccctagttgcattttatgaagcgcgagaggaccgcgcgacgggcacgcctatcgtaaatatgggt
 S  S  N  A  Q  Q  V  P  L  V  A  F  Y  E  A  R  E  D  R  A  T  G  T  P  I  V  N  M  G
gtagctCctagtctttttctggcagatatagttggtggcaagaagcattaatccatgaaattgttcatcacgttacaggctctagt
 V  A  P  S  L  F  S  G  R  Y  S  W  W  Q  E  A  L  I  H  E  I  V  H  H  V  T  G  S  S
Gatactcatgaagaaaataagcaagggcctactgaaattttagctcaaatggtcgcggcggaacttcattgggcgataccaacctttt
 D  T  H  E  E  N  K  Q  G  P  T  E  I  L  A  Q  M  V  A  A  E  L  H  W  A  I  P  T  F
aaaggataTtcagatcctgcgagggtcgaagcgatacaagagcgcgatttccactccttgttgaatatgttccagagacacggcagt
 K  G  Y  S  D  P  A  R  V  E  A  I  Q  E  R  D  F  H  S  L  L  N  M  F  Q  R  H  G  S
gaattaggctttctgttcaccagattagctacgattgccaaaggtaagaaagcttcgcctgacttcggcaccctgacctcttttgc
 E  L  G  F  L  F  T  R  L  A  T  I  A  K  G  K  K  A  S  P  D  F  G  T  L  T  S  F  C
tcggaaggtattagcagttttcctaaatatcccgatcacgatgatgatttcaacgggggcggcgccttttttctccctagcgctagc
 S  E  G  I  S  S  F  P  K  Y  P  D  H  D  D  D  F  N  G  G  G  A  F  F  L  P  S  A  S
Gccgacagttcagttgaatgcacttttgatgtactaaatcgaatcgagcctgttgatgactcaattaaatttgaaggggggaatttg
 A  D  S  S  V  E  C  T  F  D  V  L  N  R  I  E  P  V  D  D  S  I  K  F  E  G  G  N  L
ctaattaaaaatgacttcaaaaacctaaatttacgtgttgcacagcttagctttttgaacgcaaaaaaaggtagcggattttacaga
 L  I  K  N  D  F  K  N  L  N  L  R  V  A  Q  L  S  F  L  N  A  K  K  G  S  G  F  Y  R
aaaaattgggattcttggaaatcctggtatcaagcttcttcatggaagaatgggctcaattccggtctatatgagctcgctcccatg
 K  N  W  D  S  W  K  S  W  Y  Q  A  S  S  W  K  N  G  L  N  S  G  L  Y  E  L  A  P  M
Acccagacaacgcccttgaagacaagctgggttaactgctctaacatgatcgatgaaattataacacacttaaagcagccacctttg
 T  Q  T  T  P  L  K  T  S  W  V  N  C  S  N  M  I  D  E  I  I  T  H  L  K  Q  P  P  L
Cctttgctggacttcaacaacctcaatggggaagaccaagacattctgatggaaaataaccttcgaaggccaaacctggaggcattc
 P  L  L  D  F  N  N  L  N  G  E  D  Q  D  I  L  M  E  N  N  L  R  R  P  N  L  E  A  F
Aacagggctgtcaagagtttacagaacgcatcagcaattgagagcattcttaaaaatctcctgccatgtctgcccctggccacggcc
 N  R  A  V  K  S  L  Q  N  A  S  A  I  E  S  I  L  K  N  L  L  P  C  L  P  L  A  T  A
Gcacccacgcgacatccaatccatatcaaggacggtgactggaatgaattccggaggaaactgacgttctatctgaaaacccttgag
 A  P  T  R  H  P  I  H  I  K  D  G  D  W  N  E  F  R  R  K  L  T  F  Y  L  K  T  L  E
aatgcgcggctcaacagacgactttgagcctcgcgatcttttagctcgag
 N  A  Q  A  Q  Q  T  T  L  S  L  A  I  F  END L  E
                                              XhoI
```

Figure 1S. Nucleotide and amino acid sequences of AIP56-IL3 construct. AIP56 sequence is indicated in black, IL3 in purple, pET28a+ and histidines tag in red, TEV cleavage site in green, NdeI restriction site in blue and Sac I restriction site in orange.

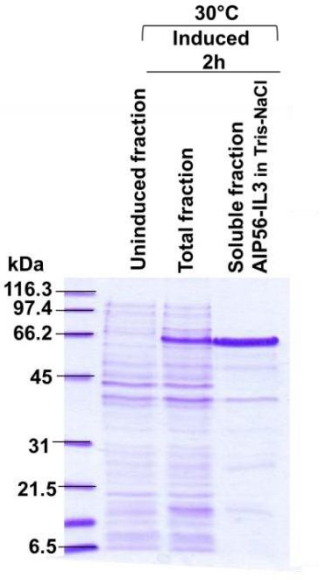

Figure 2S. Soluble refolded AIP56-IL3. Culture of the *E. coli* BL21 (DE3) competent cells transformed with plasmid pETAIP56-IL3 were grown at 30ºC, with shaking (150 rpm). When the culture reached an OD of 0.6 at 600 nm, recombinant protein expression was induced with 0.5 mM IPTG and incubated for 2h. AIP56-IL3 were isolated from *E. coli* cell pellets, solubilized, dialysed against Tris-NaCl and analyzed by Coomassie Blue stained 12% acrylamide SDS-PAGE. The overexpressed protein band at ~60.4 kDa is the protein of interest.

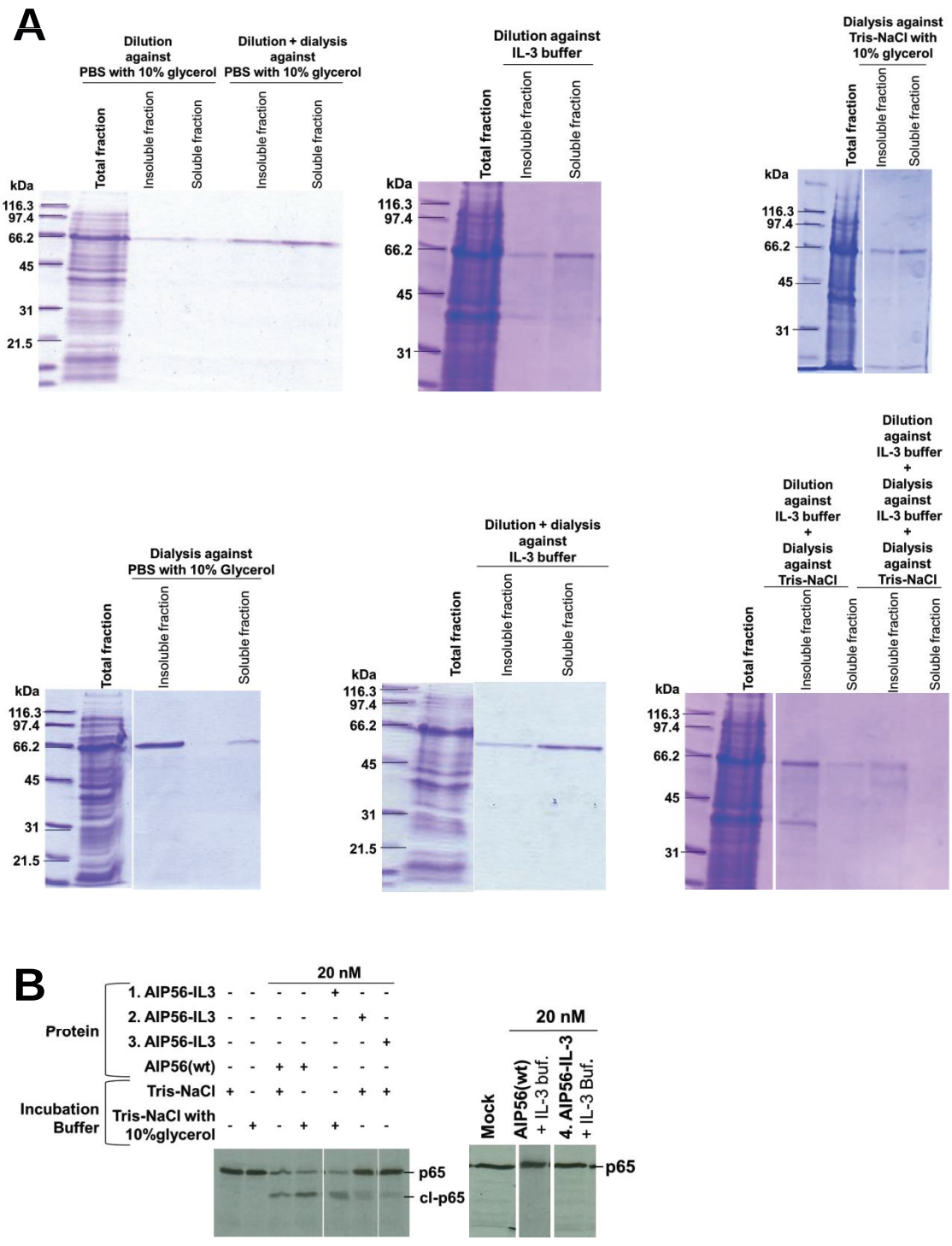

Figure 3S. Analysis of the refolding process. A) Denatured AIP56-IL3 was refolded by dialysis or dilution and dialysis against PBS with 10 % glycerol, Tris-NaCl, Tris-NaCl with 10 % glycerol or IL-3 Buffer (indicated on top of the gel). The insoluble and soluble fraction of refolded AIP56-IL3 was analyzed by Coomassie-blue stained 12% acrylamide SDS-PAGE. Molecular weight of expressed protein is ~60.4 kDa. It was also assessed whether the addition of glycerol to Tris-NaCl refolding buffer improved the refolding process [138]. However, the results showed that its effect does not significantly improve the refolding process, namely the amount of soluble protein produced as well as p65 cleavage efficiency. Therefore, its addition was discarded. B) Catalytic activity of AIP56-IL3. Incubation of in vitro translated ^{35}S-labeled sbp65Rel, in presence of the IL-3 buffer (IL-3 buf.), with AIP56 in Tris-NaCl and AIP56-IL3 in Tris-NaCl, refolded in conditions indicated as 1, 2 or 3 (see below) resulted in p65 cleavage. When AIP56-IL3 was dialyzed against IL-3 Buffer

(indicated as 4 in the blot at right) no cleavage was observed. The mix was incubated for 2 hours at 22°C with 20 nM of the indicated protein and the p65 cleavage assessed by autoradiography. The position of full length (p65) and cleaved (cl-p65) p65 are indicated.

1. Dialysis against Tris-NaCl with 10 % glycerol
2. Dilution against IL-3 buffer (IL-3 Buf.) and dialysis against Tris/NaCl
3. Dilution against IL-3 buffer and dialysis against IL-3 buffer and dialysis against Tris-NaCl
4. Dilution and dialysis against IL-3 buffer

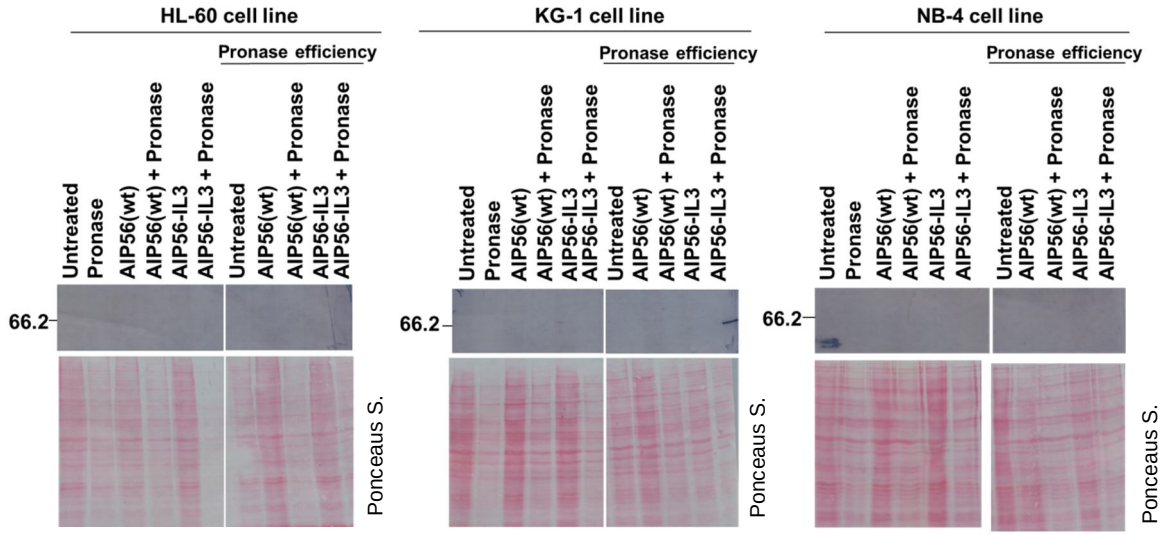

Figure 4S. Internalization assays of AIP56-IL3. AML cells were incubated with 200 nM AIP56 (11.2 µg/mL) or AIP56-IL3 (12 µg/mL) for 30 min on ice, transferred to 37ºC for 10 min and transferred again to ice for 5 min. Cells were washed twice with ice-cold PBS and incubated, when indicated, for 10 min on ice with Pronase E. Pronase E was inactivated with PMSF and the cells were washed twice with ice cold PBS. Cells untreated, treated with pronase E or treated with protein but without incubation with pronase E were used as controls. Internalized AIP56 was detected by Western blotting. In order to check the efficiency of Pronase E, a similar protocol was applied to cells where the 10 min internalization step at 37ºC has been replaced by 10 min incubation on ice to prevent internalization.